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(54) Title: MODIFIED POLYNUCLEOTIDES FOR REDUCING OFF-TARGET EFFECTS IN RNA INTERFERENCE

(57) Abstract: Methods and compositions for performing RNA interference with decreased off-target effects are provided. The methods and compositions permit effective and efficient applications of RNA interference to applications such as diagnostics and therapeutics through the use of modifications to the siRNA. Uniquely modified siRNAs have been developed that reduce off-target effects incurred in gene-silencing. The modifications comprise 2'-O-alkyl or mismatch modification(s) at specific positions on the sense and/or antisense strands.



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**TITLE**

MODIFIED POLYNUCLEOTIDES FOR REDUCING OFF-TARGET EFFECTS IN  
RNA INTERFERENCE

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**FIELD OF THE INVENTION**

The present invention relates to the field of modified polynucleotides.

**BACKGROUND**

10 Gene knockdown by RNA-induced gene silencing is presently believed to implicate a minimum of three different levels of control: (i) transcription inactivation (siRNA-guided DNA and histone methylation); (ii) small interfering RNA (siRNA)-induced mRNA degradation; and (iii) siRNA-induced transcriptional attenuation. The RNA interference (RNAi) generated by siRNA can be long lasting and effective  
15 over multiple cell divisions. Therefore, RNAi represents a potentially valuable tool that can be useful in gene function analysis, drug target validation, pathway analysis, and disease therapeutics.

Recent studies into the mechanism of RNAi-mediated transcript degradation  
20 pathway have revealed a number of key components in this pathway. A Type III RNase called Dicer processes long ds RNA into siRNA (19-23 bp duplexes) that subsequently partner with the RNA Interfering Silencing Complex (RISC) to mediate the degradation of target transcripts in a sequence specific manner. This phenomenon has been observed in a diverse group of organisms. Unfortunately, initial attempts to  
25 use long dsRNA to induce RNAi in mammalian cells met with only limited success due to induction of the interferon response, which results in a general, as opposed to targeted, inhibition of protein synthesis.

More recently, it has been shown that when short synthetic siRNAs are  
30 introduced into mammalian cells in culture, sequence-specific degradation of target mRNA can be achieved without inducing an interferon response. These short duplexes, can act catalytically at sub-molar concentrations to cleave greater than 95% of the target mRNA in a cell. A description of the mechanisms for siRNA activity, as well as some of its applications is provided in Provost *et al.*, Ribonuclease Activity

and RNA Binding of Recombinant Human Dicer, *E.M.B.O.J.*, 2002 Nov., 1, 21(21): 5864 –5874; Tabara *et al.*, The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1 and a DexH-box Helicase to Direct RNAi in *C. elegans*, *Cell* 2002, June 28, 109(7):861-71; Ketting *et al.*, Dicer Functions in RNA Interference and in Synthesis  
5 of Small RNA Involved in Developmental Timing in *C. elegans*, *Genes and Development*, 2001, 15(20):2654-9; and Martinez *et al.*, Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi, *Cell* 2002, Sept. 6, 110(5):563.

Despite the promise of RNAi, four main issues including functionality,  
10 specificity, delivery methods, and stability, must be addressed when working with siRNA. Specificity refers to the ability of a particular siRNA to silence a desired target without altering the expression of other genes, and recent studies have shown that “off-targeting” (*i.e.*, the knockdown of targets other than the intended target) is much more extensive in RNAi than originally predicted (see Jackson, A.L. *et al.*  
15 (2003) “Expression profiling reveals off-target gene regulation by RNAi” *Nature Biotechnology* 21:635-7).

As off-target effects can induce undesirable phenotypes, new methods and compositions that minimize, alter, or eliminate off-target effects are considered  
20 indispensable for siRNA to become an efficacious research and therapeutic tool. The present invention addresses the issue of specificity by providing modifications to siRNA that can either increase or alter siRNA specificity.

## SUMMARY OF THE INVENTION

25 The present invention is directed to compositions and methods for performing RNA interference. In general the siRNA chemical modifications described herein affect a critical property of the molecules: specificity. Modifications that affect specificity are particularly advantageous in research and therapeutic applications where specificity is critical. A distinct combination of modifications (and derivatives  
30 of that modification pattern) that substantially improve RNAi applications is disclosed and is applicable in the design of optimum silencing reagents.

According to a first embodiment, the present invention is directed to a double stranded ribonucleotide comprising:

- a. a sense strand, wherein said sense strand comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
  - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense strand, wherein said antisense strand comprises
  - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated on the 5' carbon of the sugar moiety, and
  - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification,

wherein said sense strand and said antisense strand are capable of forming a duplex of 18–24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

According to a second embodiment, the present invention is directed to a unimolecular siRNA capable of forming a hairpin siRNA, said unimolecular siRNA comprising:

- a. a sense strand comprising a sense region, wherein said sense region comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
  - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense strand comprising an antisense region, wherein said antisense region comprises



- i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated on the 5' carbon, and
  - 5 ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification,
  - c. a loop region, wherein said loop region is located between said sense region and said antisense region.
- 10 wherein said sense strand and said antisense strand are capable of forming a duplex of 18- 24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the duplex region, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense
- 15 nucleotide is the 5' most nucleotide of the antisense strand of the duplex region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

According to a third embodiment, the present invention is directed to a method

20 for minimizing off-target effects, said method comprising exposing an siRNA to a target nucleic acid or to a cell that is expressing or is capable of expressing said target nucleic acid, wherein said siRNA comprises:

- a. a sense strand, wherein said sense strand comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
  - 25 ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- 30 b. an antisense strand, wherein said antisense strand comprises
  - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated, and

- ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification,

wherein said sense strand and said antisense strand are capable of forming a duplex of 18–24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

According to a fourth embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing a unimolecular siRNA (that is capable of forming a hairpin) to a target nucleic acid or to a cell that is expressing or is capable of expressing said target nucleic acid, wherein said unimolecular siRNA comprises:

- a. a sense strand comprising a sense region, wherein said sense region comprises

- i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
- ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and

- b. an antisense strand comprising an antisense region, wherein said antisense region comprises

- i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated, and
- ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification, and

- c. a loop region, wherein said loop region is located between said sense region and said antisense region,

wherein said sense strand and said antisense strand are capable of forming a duplex of 18-24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most  
5 nucleotide of the sense strand of the duplex region, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand of the duplex region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

10

According to a fifth embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing two or more (*i.e.* a pool of) siRNA to a target nucleic acid or to a cell that is expressing or is capable of expressing said target nucleic acid, wherein said siRNAs comprise an antisense strand  
15 and a sense strand, wherein:

a. a sense strand, wherein said sense strand comprises

- i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
- ii. a second 5' sense nucleotide, wherein said second 5' sense  
20 nucleotide comprises a second 2'-O-alkyl modification; and

20

b. an antisense strand, wherein said antisense strand comprises

- i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated, and
- ii. a second 5' antisense nucleotide, wherein said second  
25 5' antisense nucleotide comprises a third 2'-O-alkyl modification,

25

wherein said sense strand and said antisense strand are capable of forming a duplex of 18-24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most  
30 nucleotide of the sense strand, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

30

According to a sixth embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing two or more (*i.e.* a pool of) unimolecular siRNAs (that are capable of forming a hairpin) to a target nucleic acid or to a cell that is expressing or is capable of expressing said target nucleic acid, wherein said unimolecular siRNA comprises an antisense region and a sense region, wherein:

a. said sense region comprises

- i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
- ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and

b. said antisense region comprises

- i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated, and
- ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification,

c. a loop region, wherein said loop region is located between said sense region and said antisense region.

wherein said sense region and said antisense region are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein said duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense region, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense nucleotide is the 5' most nucleotide of the antisense region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

For a better understanding of the present invention together with other and further advantages and embodiments, reference is made to the following description taken in conjunction with the examples, the scope of the which is set forth in the appended claims.

## BRIEF DESCRIPTION OF THE FIGURES

The preferred embodiments of the present invention have been chosen for purposes of illustration and description but are not intended to restrict the scope of the invention in any way. The benefits of the preferred embodiments of certain aspects of the invention are shown in the accompanying figures, wherein:

**Figure 1** depicts the relationship between positioning of one embodiment of the invention's modifications in duplexes that are longer than 25 bp. Optimal design for the molecules ensures that following Dicer digestion, the final functional duplex contains the depicted invention's modification pattern. Note, in the initial Dicer substrate, the sense and/or antisense strand can have 3' overhangs. Alternatively, both termini can be blunt ended.

**Figure 2** depicts the relationship between positioning of one embodiment of the invention's modifications in hairpins. Optimal design for the molecules ensures that following Dicer digestion, the final functional duplex contains the depicted invention's modification pattern. Note, in the initial Dicer substrate, the free (open) end of the molecule can be blunt ended or contain a 3' overhang. Furthermore, the unimolecular molecule can be organized in either a 5' antisense-loop-sense or 5' sense-loop-antisense orientation.

**Figure 3** illustrates an outline of the 2'-ACE RNA synthesis cycle.

**Figure 4** Illustrates the structure of a preferred 2'-ACE protected RNA immediately prior to 2'-deprotection.

**Figures 5A and 5B** depict the relationship between modification and function for 2'-O-methylated SEAP-2217 siRNA. The figures demonstrate the effect on gene silencing of single base (black bars) and paired (gray) 2'-O-methyl modifications of the sense strand (**Figure 5A**) and antisense strand (**Figure 5B**) of SEAP-2217. The X-axis represents the relative position of the modification along each siRNA strand (5'→3'). The Y-axis represents the percent expression relative to controls.

**Figures 6A'–6F** show the effects of 2'-O-methylation with and without 5' phosphorylation on the antisense strands of six different luciferase-specific siRNA (luc 8, 18, 56, 58, 63, and 81). "S" = sense strand. "AS" = antisense strand. "\*" indicates 2'-O-methylation at positions 1 and 2 of the designated strand. "p" indicates 5' phosphorylation of the designated strand. The Y-axis represents the % expression compared to control (untransfected) cells. "Control" = mock transfected cells.

**Figure 7** depicts a microarray expression profile (a heatmap) generated in cells treated with IGF1R-73 targeting siRNA in unmodified (top) and modified (bottom) forms. The modification pattern includes 2'-O-methyl modification of positions 1 and 2 on both the sense and antisense strands, plus phosphorylation of carbon 5 of the ribose ring of the 5' terminal antisense nucleotide. (IGF1R-73: 5'-UGCUGACCUCUGUUACCUC-3', sense)(SEQ. ID NO. 1)

**Figures 8A and 8B** depict heatmaps of cells treated with siRNA targeting: (A) MAPK14, and (B) MPHOSPH1. Duplexes MAPK14-193 and MPHOSH1-202 are either unmodified (top), sense strand modified with 2'-O-methyl modification of positions 1 and 2 (second from top), antisense strand modified with 2'-O-methyl modification of positions 1 and 2 (third from top), or both strands modified with 2'-O-methyl modifications of positions 1 and 2 (bottom of each heat map). All duplexes tested contain a phosphate group on carbon 5 of the ribose ring of the 5' terminal antisense nucleotide. Arrows indicate the position and relative levels of silencing of the target. (MPHOS1-202: 5'-GACAUGCGAAUGACACUAG-3' (SEQ. ID NO. 2); Mapk14-193: 5'-CCUACAGAGAACUGCGGUU-3' (SEQ. ID NO. 3), sense strand.

**Figure 9** depicts a summary of the number of off-targets for eight different siRNA targeting 4 separate targets MAPK14, MPHOSPH1, PTEN, and IGF1R. The pattern of siRNA chemical modification associated with each duplex is shown at the top of each column. In addition to (1) no 2'-O-methyl modifications, (2) 2'-O-methyl modifications on positions 1 and 2 of the antisense strand, (3) 2'-O-methyl modifications on positions 1 and 2 of the sense strand, or (4) 2'-O-methyl modifications on positions 1 and 2 of both strands, all duplexes tested contain a phosphate group on carbon 5 of the ribose ring of the 5' terminal antisense nucleotide.

Sense strand sequences of molecules in this figure include: IGF1R-73: 5' UGCUGACCUCUGUUACCUC-3' (**SEQ. ID NO. 4**), Mapk14-193: 5' CCUACAGAGAA CUGCGGUU-3' (**SEQ. ID NO. 5**), Mapk14-153: 5' GUCAUCAGCUUUGUGCCAC-3' (**SEQ. ID NO. 6**), MPHOS1-202:5' 5 GACAUGCAGAAUGACACUAG-3' (**SEQ. ID NO. 7**), MPHOS1-203: 5' AGAGGAACU CUCUGCAAGC-3' (**SEQ. ID NO. 8**), PTEN 213: 5' UGGAGGGGAAUGCUCAGAA-3' (**SEQ. ID NO. 9**) and PTEN 214: 5' UAAAGAUGGCACUUUCCCG-3' (**SEQ. ID NO. 10**), IGF1R-73: UGCUGACCUCUGUUACCUC-3' (**SEQ. ID NO. 11**), IGF1R-71: 5' 10 GCUCACGGUCAUUACCGAG-3' (**SEQ. ID NO. 12**)

**Figure 10a** depicts a heatmap showing the results of a chemical modification walk across the MAPK14-153 siRNA. Duplexes carry single modifications or paired 2'-O-methyl modifications of various positions of the siRNA antisense strand in combination with 2'-O-methyl modifications on positions 1 and 2 of the sense strand and a phosphate group on the 5' carbon of the ribose ring of the first antisense (terminal) nucleotide. Mapk14-153: 5' GUCAUCAGCUUUGUGCCAC-3' (**SEQ. ID NO. 13**), sense strand. The letters D-R describe the following molecules:

- D:** unmodified
- 20 **E** 2'-O-methyl modification of positions 1 and 2 of the AS strand;
- F:** 2'-O-methyl modification of positions 1 and 2 of the AS strand without modification of the sense strand;
- G:** 2'-O-methyl modification of positions 2 and 3 of the AS strand;
- H:** 2'-O-methyl modification of positions 3 and 4 of the AS strand;
- 25 **I:** 2'-O-methyl modification of positions 4 and 5 of the AS strand;
- J:** 2'-O-methyl modification of positions 5 and 6 of the AS strand;
- K:** 2'-O-methyl modification of positions 6 and 7 of the AS strand;
- L:** 2'-O-methyl modification of positions 7 and 8 of the AS strand;
- M:** 2'-O-methyl modification of positions 8 and 9 of the AS strand;
- 30 **N:** 2'-O-methyl modification of positions 9 and 10 of the AS strand;
- O:** 2'-O-methyl modification of positions 10 and 11 of the AS strand;
- P:** 2'-O-methyl modification of position 1 of the AS strand;
- Q:** 2'-O-methyl modification of position 2 of the AS strand; and
- R:** 2'-O-methyl modification of positions 1, 2, 11, and 12 of the AS strand

**Figure 10b** depicts a heatmap demonstrating how position 2 is a critical nucleotide in determining off-target gene modulation. A) siRNA targeting three different genes (MAPK14, KNTC2, and STK6) were examined for off-target effects by microarray analysis when the duplex was unmodified (top of each set of heat maps), modified with 2'-O-methyl groups on positions 1 and 2 of the sense strand (second from top in each set of heat maps), modified with 2'-O-methyl groups on positions 1 and 2 of the sense strand plus 2'-O-methyl modifications on position 1 of the antisense strand (third from top of each set of heatmaps), modified with 2'-O-methyl groups on positions 1 and 2 of the sense strand plus 2'-O-methyl modifications on position 2 of the antisense strand (fourth from top of each set of heatmaps), and modified with 2'-O-methyl groups on positions 1 and 2 of the sense strand plus 2'-O-methyl modifications on positions 1 and 2 of the antisense strand (fifth from top of each set of heatmaps). All duplexes in this study contained a phosphate group on the 5' carbon of the ribose ring of the first antisense (terminal) nucleotide. Sense strand sequences used in this figure include: KNTC2: 5' GGCUUCCUUACAAGGAGAU-3' (**SEQ. ID NO. 14**), Mapk14-193: 5' CCUACAGAGAACUGCGGUU-3' (**SEQ. ID NO. 15**), STK6: CGGGUCUUGUGUCCUCAA-3' (**SEQ. ID NO. 16**).

**Figure 11** compares the effects of chemical modification with selective basepair mismatches on siRNA (MAPK14-153) induced off-target effects (Mapk14-153: 5'GUCAUCAGCUUUGUGCCAC-3' (**SEQ. ID NO. 17**), sense) The top **eight** heat maps represent duplexes that are: unmodified (top), or contain 2'-O-methyl modifications at positions 1 and 2, 2 and 3, 3 and 4, 4 and 5, 5 and 6, 6 and 7, or 7 and 8 of the antisense strand. All of the duplexes contain a phosphate group on the 5' carbon of the ribose ring of the first antisense (terminal) nucleotide. The lower half of the heatmap represents siRNA that have basepair mismatches (between the antisense strand of the siRNA and the target molecule) incorporated into the siRNA duplexes. All of the duplexes contain a phosphate group on the 5' carbon of the ribose ring of the first antisense (terminal) nucleotide.

**Figures 12a-d** demonstrate target independent, sequence specific siRNA toxicity. **12a:** . HeLa cells were transfected with one of 90 different siRNA targeting DBI (NM\_020548, position 202-291). Data are displayed according to siRNA position in walk.



Dotted (horizontal) line represents the 75% viability threshold. Boxed areas indicate toxic siRNA with sequence similarity. Toxicity data (gray bars) are superimposed on DBI mRNA expression data (black bars) for the same siRNA set. **12b:** HeLa cells were transfected with one of 48 functional (>70% silencing) siRNA targeting 12 different genes. Data is sorted based on the level of siRNA-induced toxicity. Non-toxic siRNA (gray bars), toxic siRNA (black bars). **12c:** HeLa cells transfected with a subset of the toxic and non-toxic siRNA from (b) targeting either MAPK1 (MEK1) or MAPK2 (MEK2). Toxicity data (gray bars) are presented alongside mRNA expression data (black bars). Data show that there is no correlation between the level of silencing and toxicity. **12d:** Dilution studies showing the effects of toxic siRNA (MAP2K2-3, SRD5A1-1, SRD5A1-3 and SRD5A2-3) in HeLa cells at varying concentrations. Values for toxicity represent the average of three independent experiments (each performed in triplicate). Error bars depict standard deviation from the mean. For experimental protocols: 72h hours after transfection, 25 microliters of Alamar Blue dye were added to wells containing cells in 100 microliters of media. Cells were then incubated (0.5 hrs) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The fluorescence was subsequently measured on a Perkin Elmer WallacVector2 1420 multi-label counter with excitation at 540 nm and emission at 590nm. The results presented in **Figure 12** are an average of nine data points coming from three independent experiments performed on different days. For the purpose of this study, siRNAs were defined as toxic when the results from nine different experiments (taking into account standard deviations) showed cell viability was below 75%. For comparative gene expression levels, mRNA was quantitated using Quantigene® Kits (Genospectra, Fremont, CA) for branched DNA (bDNA) assay according to manufacturer instructions. Level of mRNA of GAPDH (a housekeeping gene) was used as a reference.

**Figures 13a-c.** Sequence dependence of siRNA toxicity. HeLa cells were transfected with functional siRNA containing **13a:** AAA/UUU, **13b:** GCCA/UGGC, or **13c:** no toxic motifs. Motif-containing siRNA exhibit heightened incidence of toxicity. Values for toxicity represent the mean of three independent experiments (each performed in triplicate). Error bars depict standard deviation from the mean.

**Figures 14a-l** illustrate siRNA-induced toxicity mediated through the RNAi pathway.

**14a:** Diagram of experimental procedures used in eIF2C2/Ago2 knockdown experiments. "T1" and "T2" represent "transfection 1" and transfection 2", respectively. Control and test siRNA were transfected in at 10nM in each

transfection, **14b-14i:** Control experiments demonstrating that knockdown of the eIF2C2 gene product disables the RNAi pathway (**b, d, f, and h** - depicts EGFP expression levels; **c, e, g, and i** - show comparative Hoechst 33342 staining). Study shows that if one disables the RNAi pathway with eIF2C2 siRNA, then subsequent addition of targeting siRNA fail silence their target. **14j:** Graph showing the effects of eIF2C2 knockdown on siRNA toxicity; **14k:** Graph showing the effect of truncating toxic siRNA by 2 nucleotides (19mer→17mer) on siRNA toxicity. **14l:** Graph showing the effects that chemical modifications of toxic siRNA have on siRNA toxicity. **14m.** Toxicity of 37 luciferase targeting sequences in modified and unmodified forms; **14n.** Graph showing the level of silencing of luciferase-targeting sequences in modified and unmodified forms. Values for toxicity represent the average of three independent experiments (each performed in triplicate). Error bars depict standard deviation from the mean. Regular and fluorescent microscopy was used to obtain data on cellular and nuclei morphology. Live cells were stained with cell-permeable nuclear fluorescent dye Hoechst 33342 (2 microg/ml, 15 minutes at 37°C, Molecular Probes). Pictures were taken using Leica DML fluorescent microscope InSight CCD camera and SPOT 3.5 software.

**Figure 15** illustrates a 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)silyl-2'-O-*bis*(2-acetoxyethyl)orthoformyl-3'-O-(N,N-diisopropyl)methyl phosphoramidite. B is a nucleoside base such as, for example, adenosine, guanosine, cytidine, or uracil; Z is a protecting group for the exocyclic amine (isobutyryl for A and G, acetyl for C).

**Figure 16** illustrates a 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)-silyl-2'-O-methyl-3'-O-(N,N-diisopropyl)methyl phosphoramidite. B is a nucleoside base such as, for example, adenosine, guanosine, cytidine, or uracil; Z is a protecting group for the exocyclic amine (isobutyryl for A and G, acetyl for C).

**Figure 17** illustrates an N,N-diisopropylamino-*bis*(2-cyanoethyl) phosphoramidite.

**Figure 18** shows heat maps generated from HeLa cells treated with cyclophilin B (18a-18c) or MAP2K1 (18d-18f) duplexes and pools in unmodified and modified forms. “N” = normal, unmodified. “OTP” = modified. Results demonstrate that addition of chemical modifications of the invention to individual duplexes generally reduces the number of off-targeted genes by 50% or more (see 18a, 18b, 18d, 18e). Combination of modifications of the invention with pooling reduces the number of genes that are down-regulated by > 2 fold by over 90% (see 18c, 18f).

## DETAILED DESCRIPTION

The present invention will now be described in connection with preferred embodiments. These embodiments are presented to aid in an understanding of the present invention and are not intended, and should not be construed, to limit the invention in any way. All alternatives, modifications and equivalents that may become apparent to those of ordinary skill upon reading this disclosure are included within the spirit and scope of the present invention.

This disclosure is not a primer on compositions and methods for performing RNA interference. Basic concepts known to those skilled in the art have not been set forth in detail.

The present invention is directed to compositions and methods for performing RNA interference, including siRNA-induced gene silencing. Through the use of the present invention, modified polynucleotides, and derivatives thereof, one may improve the efficiency of RNA interference applications.

Unless explicitly stated otherwise, or implicit from content, the following terms and phrases include the meanings provided below:

### ALKYL

The term “alkyl” refers to a hydrocarbyl moiety that can be saturated or unsaturated. It may comprise moieties that are linear, branched and/or cyclic.

Exemplary alkyl groups include but are not limited to moieties such as, for example, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl,

undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, eicosyl and alkyl groups of higher number of carbons, as well as 2-methylpropyl, 2-methyl-4-ethylbutyl, 2,4-diethylpropyl, 3-propylbutyl, 2,8-dibutyldecyl, 6,6-dimethyloctyl, 6-propyl-6-butyloctyl, 2-methylbutyl, 2-methylpentyl, 3-methylpentyl, 2-ethylhexyl, isopropyl, isobutyl, isopentyl, *etc.* The term alkyl also encompasses alkenyl groups, such as vinyl, allyl, aralkyl and alkynyl groups. Unless otherwise specified, alkyl groups are not substituted.

The preferred alkyl group for a 2' modification is a methyl group with an O-linkage to the 2' carbon of a ribosyl moiety, *i.e.*, a 2'-O-alkyl that comprises a 2'-O-methyl group. A preferred 2'-O-methyl group is unsubstituted: -O-CH<sub>3</sub>

## 2'-O-ALKYL MODIFIED NUCLEOTIDE

The phrase "2'-O-alkyl modified nucleotide" refers to a nucleotide unit having a sugar moiety, for example a deoxyribosyl moiety that is modified at the 2' position such that an oxygen atom is attached both to the carbon atom located at the 2' position of the sugar and to an alkyl group. In various embodiments, the alkyl moiety consists essentially of carbons and hydrogens. A particularly preferred embodiment is one wherein the alkyl moiety is a methyl moiety.

## ANTISENSE STRAND

The phrase "antisense strand" as used herein, refers to a polynucleotide or region of a polynucleotide that is substantially (*i.e.*, 80% or more) or 100% complementary to a target nucleic acid of interest. An antisense strand may be comprised of a polynucleotide region that is RNA, DNA or chimeric RNA/DNA. For example, an antisense strand may be complementary, in whole or in part, to a molecule of messenger RNA, an RNA sequence that is not mRNA (*e.g.*, tRNA, rRNA and hnRNA) or a sequence of DNA that is either coding or non-coding. The phrase "antisense strand" includes the antisense region of polynucleotides that are formed from two separate strands, as well as unimolecular siRNAs that are capable of forming hairpin structures. The phrases "antisense strand" and "antisense region" are intended to be equivalent and are used interchangeably. The antisense strand can be modified with a diverse group of small molecules and/or conjugates.

## 2' CARBON MODIFICATION

The phrase "2' carbon modification" refers to a nucleotide unit having a sugar moiety, for example a moiety that is modified at the 2' position of the sugar subunit. A "2'-O-alkyl modified nucleotide" is modified at this position such that an oxygen atom is attached both to the carbon atom located at the 2' position of the sugar and to an alkyl group, *e.g.*, 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-isopropyl, 2'-O-butyl, 2'-O-isobutyl, 2'-O-ethyl-O-methyl (-OCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>), and 2'-O-ethyl-OH (-OCH<sub>2</sub>CH<sub>2</sub>OH). A "2' carbon sense modification" refers to a modification at the 2' carbon position of a nucleotide on the sense strand or within a sense region of polynucleotide. A "2' carbon antisense modification" refers to a modification at the 2' carbon position of a nucleotide on the antisense strand or within an antisense region of polynucleotide.

## COMPLEMENTARY

The term "complementary" refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands or regions. Complementary polynucleotide strands or regions can base pair in the Watson-Crick manner (*e.g.*, A to T, A to U, C to G), or in any other manner that allows for the formation of stable duplexes. Complementarity is typically measured with respect to a duplex region and thus excludes, for example, overhangs. A duplex region comprises a region of complementarity between two strands or between two regions of a single strand, for example, a unimolecular siRNA. Typically, the region of complementarity results from Watson-Crick base pairing.

Perfect complementarity or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand or region can hydrogen bond with each nucleotide unit of a second polynucleotide strand or region. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands or two regions can hydrogen bond with each other. For example, for two 20-mers, if only two base pairs on each strand can hydrogen bond with each other, the polynucleotide strands or regions exhibit 10% complementarity. In the same example, if 18 base pairs on each strand or each region can hydrogen bond with each other, the polynucleotide strands exhibit 90% complementarity. Substantial

complementarity refers to polynucleotide strands or regions exhibiting 80% or greater complementarity.

#### **DEOXYNUCLEOTIDE**

5           The term “deoxynucleotide” refers to a nucleotide or polynucleotide lacking an OH group at the 2’ or 3’ position of a sugar moiety, and/or a 2’,3’ terminal dideoxy, but instead having a hydrogen at the 2’ and/or 3’ carbon.

#### **DEOXYRIBONUCLEOTIDE**

10           The terms “deoxyribonucleotide” and “DNA” refer to a nucleotide or polynucleotide comprising at least one ribosyl moiety that has an H at the 2’ position of a ribosyl moiety. Preferably a deoxyribonucleotide is a nucleotide having an H at its 2’ position.

#### **DOWNSTREAM**

15           A first region or nucleotide of a strand of nucleotides is considered to be downstream of a second region, if the 5’ most portion of the first region is the closest portion of that region to the 3’ end of the second region (or nucleotide).

#### **FIRST 5’ TERMINAL ANTISENSE NUCLEOTIDE**

20           The phrase “first 5’ terminal antisense nucleotide” refers to the nucleotide of the antisense strand or region that is located at the 5’ most position of that strand with respect to the bases of the antisense strand or region that have corresponding complementary bases on the sense strand or region. Thus, in an siRNA that is made  
25 of two separate strands (*i.e.*, not a unimolecular or hairpin siRNA), it refers to the 5’ most base other than bases that are part of any 5’ overhang on the antisense strand, which may or may not be present. When the first 5’ terminal antisense nucleotide is part of a hairpin molecule, the term “terminal” refers to the 5’ most relative position within the antisense region and thus is the 5’ most nucleotide of the antisense region.

30

#### **FIRST 5’ TERMINAL SENSE NUCLEOTIDE**

          The phrase “first 5’ terminal sense nucleotide” is defined in reference to the antisense nucleotide. In molecules that are comprised of two separate strands (*i.e.*, not a unimolecular or hairpin siRNA), it refers to the nucleotide of the sense strand

that is located at the 5' most position of that strand with respect to the bases of the sense strand that have corresponding complementary bases on the antisense strand. Thus, in an siRNA that is made of two separate strands (*i.e.*, not a unimolecular or hairpin siRNA), it is the 5' most base other than bases that are part of any 5' overhang  
5 on the sense strand or region, which may or may not be present. When the first 5' terminal sense nucleotide is part of a unimolecular siRNA that is capable of forming a hairpin molecule, the term "terminal" refers to the relative position within the sense strand or region as measured by the distance from the base complementary to the first 5' terminal antisense nucleotide.

10

### FUNCTIONAL

siRNA may be divided into five (5) groups (non-functional, semi-functional, functional, highly functional, and hyperfunctional) based on the level or degree of silencing that they induce in cultured cell lines. As used herein, these definitions are  
15 based on a set of conditions where the siRNA is transfected into said cell line at a concentration of 100 nM and the level of silencing is tested at a time of roughly 24 hours after transfection, and not exceeding 72 hours after transfection. In this context, "non-functional siRNA" are defined as those siRNA that induce less than 50% (<50%) target silencing. "Semi-functional siRNA" induce 50-79% target silencing.  
20 "Functional siRNA" are molecules that induce 80-95% gene silencing. "Highly-functional siRNA" are molecules that induce greater than 95% gene silencing. "Hyperfunctional siRNA" are a special class of molecules. For purposes of this document, hyperfunctional siRNA are defined as those molecules that: (1) induce greater than 95% silencing of a specific target when they are transfected at  
25 subnanomolar concentrations (*i.e.*, less than one nanomolar); and/or (2) induce functional (or better) levels of silencing for greater than 96 hours. These relative functionalities (though not intended to be absolutes) may be used to compare siRNAs to a particular target for applications such as functional genomics, target identification and therapeutics.

30

### FUNCTIONAL DOSE

A "functional dose" refers to a dose of siRNA that will be effective at causing a greater than or equal to 95% reduction in mRNA at levels of 100 nM at 24, 48, 72, and 96 hours following administration, while a "marginally functional dose" of

siRNA will be effective at causing a greater than or equal to 50% reduction of mRNA at 100 nM at 24 hours following administration and a “non-functional dose” of RNA will cause a less than 50% reduction in mRNA levels at 100 nM at 24 hours following administration.

5

**MISMATCH**

The term “mismatch” includes a situation in which Watson-Crick base pairing does not take place between a nucleotide of a sense strand and a nucleotide of an antisense strand, where the nucleotides are flanked by a duplex comprising base pairs in the 5’ direction of the mismatch beginning directly after (in the 5’ direction) the mismatched position and in the 3’ direction of the mismatch beginning directly after (in the 3’ direction) the mismatched position. An example of a mismatch would be an A across from a G, a C across from an A, a U across from a C, an A across from an A, a G across from a G, a C across from a C, and so on. Mismatches are also meant to include an abasic residue across from a nucleotide or modified nucleotide, an acyclic residue across from a nucleotide or modified nucleotide, a gap, or an unpaired loop. In its broadest sense, a mismatch as used herein includes any alteration at a given position that decreases the thermodynamic stability at or in the vicinity of the position where the alteration appears, such that the thermodynamic stability of the duplex at the particular position is less than the thermodynamic stability of a Watson-Crick base pair at that position. Preferred mismatches include a G across from an A, and an A across from a C. A particularly preferred mismatch comprises an A across from an A, G across from a G, C across from a C, and U across from a U.

25 **NUCLEOTIDE**

The term “nucleotide” refers to a ribonucleotide or a deoxyribonucleotide or modified form thereof, as well as an analog thereof. Nucleotides include species that comprise purines, *e.g.*, adenine, hypoxanthine, guanine, and their derivatives and analogs, as well as pyrimidines, *e.g.*, cytosine, uracil, thymine, and their derivatives and analogs. Preferably, a “nucleotide” comprises a cytosine, uracil, thymine, adenine, or guanine moiety. Preferred nucleotides, unless otherwise specified (such as, for example, when specifying a 2’ modification, 5’ modification, 3’ modification, nucleobase modification, or modified internucleotide linkage), include unmodified cytosine, uracil, thymine, adenine, and guanine.



Nucleotide analogs include nucleotides having modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, and substitution of 5-bromo-uracil; and 2'-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2'-OH is replaced by a group such as an H, OR, R, halo, SH, SR, NH<sub>2</sub>, NHR, NR<sub>2</sub>, or CN, wherein R is an alkyl moiety as defined herein. Nucleotide analogs are also meant to include nucleotides with bases such as inosine, queuosine, xanthine, sugars such as 2'-methyl ribose, non-natural phosphodiester linkages such as methylphosphonates, phosphorothioates and peptides.

Modified bases refer to nucleotide bases such as, for example, adenine, guanine, cytosine, thymine, and uracil, xanthine, inosine, and queuosine that have been modified by the replacement or addition of one or more atoms or groups. Some examples of types of modifications that can comprise nucleotides that are modified with respect to the base moieties, include but are not limited to, alkylated, halogenated, thiolated, aminated, amidated, or acetylated bases, in various combinations. More specific modified bases include, for example, 5-propynyluridine, 5-propynylcytidine, 6-methyladenine, 6-methylguanine, N,N,-dimethyladenine, 2-propyladenine, 2-propylguanine, 2-aminoadenine, 1-methylinosine, 3-methyluridine, 5-methylcytidine, 5-methyluridine and other nucleotides having a modification at the 5 position, 5-(2-amino)propyl uridine, 5-halocytidine, 5-halouridine, 4-acetylcytidine, 1-methyladenosine, 2-methyladenosine, 3-methylcytidine, 6-methyluridine, 2-methylguanosine, 7-methylguanosine, 2,2-dimethylguanosine, 5-methylaminoethyluridine, 5-methyloxyuridine, deazanucleotides such as 7-deaza-adenosine, 6-azouridine, 6-azocytidine, 6-azothymidine, 5-methyl-2-thiouridine, other thio bases such as 2-thiouridine and 4-thiouridine and 2-thiocytidine, dihydrouridine, pseudouridine, queuosine, archaeosine, naphthyl and substituted naphthyl groups, any O- and N-alkylated purines and pyrimidines such as N6-methyladenosine, 5-methylcarbonylmethyluridine, uridine 5-oxyacetic acid, pyridine-4-one, pyridine-2-one, phenyl and modified phenyl groups such as aminophenol or 2,4,6-trimethoxy benzene, modified cytosines that act as G-clamp nucleotides, 8-substituted adenines and guanines, 5-substituted uracils and thymines, azapyrimidines,

carboxyhydroxyalkyl nucleotides, carboxyalkylaminoalkyl nucleotides, and alkylcarbonylalkylated nucleotides. Modified nucleotides also include those nucleotides that are modified with respect to the sugar moiety, as well as nucleotides having sugars or analogs thereof that are not ribosyl. For example, the sugar moieties  
5 may be, or be based on, mannoses, arabinoses, glucopyranoses, galactopyranoses, 4'-thioribose, and other sugars, heterocycles, or carbocycles. The term nucleotide is also meant to include what are known in the art as universal bases. By way of example, universal bases include but are not limited to 3-nitropyrrole, 5-nitroindole, or nebularine.

10

Further, the term nucleotide also includes those species that have a detectable label, such as for example a radioactive or fluorescent moiety, or mass label attached to the nucleotide.

#### 15 NUCLEOTIDE UNIT

The phrase "nucleotide unit" refers to a single nucleotide residue and is comprised of a modified or unmodified nitrogenous base, a modified or unmodified sugar, and a modified or unmodified moiety that allows for linking of two nucleotides together or a conjugate that precludes further linkage.

20

#### OFF-TARGET

The term "off-target" and the phrase "off-target effects" refer to any instance in which an siRNA or shRNA directed against a given target causes an unintended effect by interacting either directly or indirectly with another mRNA sequence, a  
25 DNA sequence or a cellular protein or other moiety. For example, an "off-target effect" may occur when there is a simultaneous degradation of other transcripts due to partial homology or complementarity between that other transcript and the sense and/or antisense strand of the siRNA or shRNA.

#### 30 OVERHANG

The term "overhang" refers to terminal non-base pairing nucleotide(s) resulting from one strand or region extending beyond the terminus of the complementary strand to which the first strand or region forms a duplex. One or both of two polynucleotides or polynucleotide regions that are capable of forming a duplex

through hydrogen bonding of base pairs may have a 5' and/or 3' end that extends beyond the 3' and/or 5' end of complementarity shared by the two polynucleotides or regions. The single-stranded region extending beyond the 3' and/or 5' end of the duplex is referred to as an overhang.

5

#### **PHARMACEUTICALLY ACCEPTABLE CARRIER**

The phrase "pharmaceutically acceptable carrier" includes, but is not limited to, compositions that facilitate the introduction of dsRNA, dsDNA, or dsRNA/DNA hybrids into a cell and includes but is not limited to solvents or dispersants, coatings, anti-infective agents, isotonic agents, and agents that mediate absorption time or release of the inventive polynucleotides and siRNAs.

10

#### **POLYNUCLEOTIDE**

The term "polynucleotide" refers to polymers of nucleotides, and includes but is not limited to DNA, RNA, DNA/RNA hybrids including polynucleotide chains of regularly and irregularly alternating deoxyribosyl moieties and ribosyl moieties (*i.e.*, wherein alternate nucleotide units have an -OH, then an -H, then an -OH, then an -H, and so on at the 2' position of a sugar moiety), and modifications of these kinds of polynucleotides wherein the attachment of various entities or moieties to the nucleotide units at any position are included. Unless otherwise specified, or clear from context, the term "polynucleotide" includes both unimolecular siRNAs and siRNAs comprised of two separate strands.

15

20

#### **POLYRIBONUCLEOTIDE**

The term "polyribonucleotide" refers to a polynucleotide comprising two or more modified or unmodified ribonucleotides and/or their analogs.

25

#### **POOLING**

The term "pooling" refers to the process whereby two or more siRNA (preferably rationally designed siRNA) targeting a single gene are combined and introduced into a cell to induce gene knockdown.

30

#### **RIBONUCLEOTIDE AND RIBONUCLEIC ACID**

The term "ribonucleotide" and the phrase "ribonucleic acid" (RNA), refer to a

modified or unmodified nucleotide or polynucleotide comprising at least one ribonucleotide unit. A ribonucleotide unit comprises an oxygen attached to the 2' position of a ribosyl moiety having a nitrogenous base attached in N-glycosidic linkage at the 1' position of a ribosyl moiety, and a moiety that either allows for  
5 linkage to another nucleotide or precludes linkage.

#### **RNA INTERFERENCE AND RNAI**

The phrase "RNA interference" and the term "RNAi" are synonymous and refer to the process by which a polynucleotide or siRNA comprising at least one  
10 ribonucleotide unit exerts an effect on a biological process. The process includes, but is not limited to, gene silencing by degrading mRNA, attenuating translation, interactions with tRNA, rRNA, hnRNA, cDNA and genomic DNA, as well as methylation of DNA with ancillary proteins.

#### **15 SECOND NUCLEOTIDE**

The term "second nucleotide" or "nucleotide number 2" refer to the second nucleotide within a duplex region on either the sense or antisense strand, counting from the 5' end of each respective strand. The nucleotide may be paired or, for example in the case of a mismatch, unpaired.

20

#### **SECOND 5' TERMINAL ANTISENSE NUCLEOTIDE**

The phrase "second 5' terminal antisense nucleotide" refers to the nucleotide that is immediately adjacent to the first 5' terminal antisense nucleotide and attached to the 3' position of the first 5' terminal antisense nucleotide. Thus, it is the second  
25 most 5' nucleotide of the antisense strand or region within the set of nucleotides for which there are complementary sense nucleotides.

#### **SECOND 5' TERMINAL SENSE NUCLEOTIDE**

The phrase "second 5' terminal sense nucleotide" refers to the nucleotide that  
30 is immediately adjacent to the first 5' terminal sense nucleotide and attached to the 3' position of the first 5' terminal sense nucleotide. Thus, it is the second most 5' nucleotide of the sense strand or region within the set of nucleotides for which there are corresponding antisense nucleotides.

**SENSE STRAND**

The phrase “sense strand” refers to a polynucleotide or region that has the same nucleotide sequence, in whole or in part, as a target nucleic acid such as a messenger RNA or a sequence of DNA. The phrase “sense strand” includes the sense  
5 region of both polynucleotides that are formed from two separate strands, as well as unimolecular siRNAs that are capable of forming hairpin structures. When a sequence is provided, by convention, unless otherwise indicated, it is the sense strand (or region), and the presence of the complementary antisense strand (or region) is implicit. The phrases “sense strand” and “sense region” are intended to be equivalent  
10 and are used interchangeably.

**siRNA OR SHORT INTERFERING RNA**

The term “siRNA” and the phrase “short interfering RNA” refer to unimolecular nucleic acids and to nucleic acids comprising two separate strands that  
15 are capable of performing RNAi and that have a duplex region that is between 18 and 30 base pairs in length. Additionally, the term siRNA and the phrase “short interfering RNA” include nucleic acids that also contain moieties other than ribonucleotide moieties, including, but not limited to, modified nucleotides, modified internucleotide linkages, non-nucleotides, deoxynucleotides and analogs of the  
20 aforementioned nucleotides.

siRNAs can be duplexes and can also comprise unimolecular polynucleotides. Such unimolecular molecules comprise regions of self-complementarity (a stem) whereby nucleotides from one region of the polynucleotide pair with another region  
25 of the polynucleotide (thus forming a duplex), and are separated by a loop. Such unimolecular molecules can vary in size and design and are referred to by a variety of names including but not limited to hairpins, short hairpin RNAs (shRNAs), microRNAs (miRNAs) and short temporal RNAs (stRNAs). The length of the stem region in these molecules can vary between 18 and 45 nucleotides in length.  
30 Similarly, the size of the loop can vary between 4 and 23 nucleotides and can comprise nucleotide, non-nucleotide, and nucleotide-non-nucleotide compositions.

When the siRNAs are hairpins, the sense strand and antisense strand are part

of one longer molecule.

#### SUBSTANTIAL COMPLEMENTARITY

Substantial complementarity refers to polynucleotide strands exhibiting 80%  
5 or greater complementarity.

#### PREFERRED EMBODIMENTS

The present invention will now be described in connection with preferred  
10 embodiments. These embodiments are presented in order to aid in an understanding  
of the present invention and are not intended and should not be construed to limit the  
invention in any way. All alternatives, modifications and equivalents that may  
become apparent to those of ordinary skill upon reading this disclosure are included  
within the spirit and scope of the present invention.

15

According to a first embodiment, the present invention is directed to a double  
stranded ribonucleotide comprising:

- a. a sense strand, wherein said sense strand comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide  
20 comprises a first 2'-O-alkyl modification, and
  - ii. a second 5' sense nucleotide, wherein said second 5' sense  
nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense strand, wherein said antisense strand comprises
  - i. a first 5' antisense nucleotide, wherein said first 5' antisense  
25 nucleotide is phosphorylated on the 5' carbon, and
  - ii. a second 5' antisense nucleotide, wherein said second 5'  
antisense nucleotide comprises a third 2'-O-alkyl modification,

25

wherein said sense strand and said antisense strand are capable of forming a duplex of  
18 –24 base pairs of nucleotides that has at least 80% complementarity over the range  
30 of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most  
nucleotide of the sense strand, said second 5' sense nucleotide is immediately  
adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense  
nucleotide is the 5' most nucleotide of the antisense strand and said second 5'

antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

5 In one embodiment, the first 5' antisense nucleotide comprises an -OH at its 2' position. In this embodiment, the first 2'-O-alkyl modification preferably comprises 2'-O-methyl, the second 2'-O-alkyl modification preferably comprises 2'-O-methyl, and the third 2'-O-alkyl modification preferably comprises 2'-O-methyl. In a preferred embodiment, the double stranded ribonucleotide of this embodiment comprises a 2'-OH on all nucleotides other than the first 5' sense nucleotide, the  
10 second 5' sense nucleotide, and the second 5' antisense nucleotide.

In another embodiment, the first 5' antisense nucleotide comprises a fourth 2'-O-alkyl modification. In this embodiment, the first 2'-O-alkyl modification comprises 2'-O-methyl, the second 2'-O-alkyl modification comprises 2'-O-methyl,  
15 the third 2'-O-alkyl modification comprises 2'-O-methyl, and the fourth 2'-O-alkyl modification comprises 2'-O-methyl. In a preferred embodiment, this embodiment further comprises a 2'-OH on all nucleotides other than on the first 5' sense nucleotide, the second 5' sense nucleotide, the first 5' antisense nucleotide, the said second 5' antisense nucleotide.

20

The authors recognize that the second position of the antisense (and sense) strand of siRNAs that are between 18-24 bp in length, is (unlike any other base or basepair in the siRNA) a key position in mediating the silencing of targets other than the intended target (*i.e.*, off-targets) and that modifications/alterations of this position  
25 can be used to eliminate off-target effects generated by any siRNA. As shown in Example 8 of this document, chemical modification of position 2 effectively eliminates off-target effects generated by that strand as measured by microarray analysis. Similarly, Example 9 demonstrates how addition of basepair mismatches at position 2 (and other positions) can dramatically alter the pattern of off-target effects.  
30 The ability to shift or alter off-target effects is particularly valuable in instances where down regulation of one or more off-target genes induces an undesirable phenotype. Evidence that such off-target induced phenotypes exist and can be eliminated by alterations at position 2 of the AS strand is provided in Example 10.

Knowledge of the importance of position 2 (on either the sense and/or antisense strand) enables one to effectively eliminate off-target effects by a variety of strategies. For instance, addition of 2' modification (such as a 2'-O-alkyl modification) at this position on *e.g.* the antisense strand, can eliminate the off-target effects attributable to this strand. Similarly, one can substitute a base at position 2 of, *e.g.*, the antisense strand, such that a mismatch now exists between the intended target mRNA and the antisense strand of the siRNA. While a single basepair mismatch (or a modification) at this position will not dramatically alter the ability of this siRNA to silence the intended target, it will alter the ability of the siRNA to silence off-targets. It should be noted that basepair mismatches at positions other than position 2 of *e.g.* the antisense strand (*e.g.* at positions 3, 4, 5, 6, 7, or 8 in the antisense strand of an siRNA duplex where numbering references a location with respect to the 5 terminus of the antisense strand, with position 2 being the nucleotide that is adjacent to the 5'-most nucleotide on the antisense strand) can also reduce, eliminate, or alter specific off-target effects. Suitable mismatches include, but are not limited to A-G pairings, A-A pairings, G-G pairings, C-C pairings, and U-U pairings. Moreover, the position of the mismatch is preferably at position 3, 4, 5, 6, 7, or 8 of the antisense or sense strand. Most preferably, the position of the mismatch is at position 2 of the antisense or sense strand. It should be noted that while use of basepair substitutions such as this can eliminate the original set of off-targets, new off-targets (resulting from complementarity to a new set of genes) can result. For this reason, it is more preferable that the chemical modifications described in this invention are added to the siRNA that eliminate or minimize all off-target effects. Thus modifications including but not limited to: 1) chemical modifications of the base, sugar, or internucleotide linkage of nucleotide number two of the sense and/or antisense strand; (2) nucleotide or nucleotide pair alterations at position 2, 3, 4, 5, 6, 7, or 8 of the sense and/or antisense strand of an siRNA, including substitution of a base or base pair such that a mismatch is generated between a potential off-target mRNA and either the sense and/or antisense strand of the siRNA; (3) nucleotide or nucleotide pair alterations at position 2 of the sense and/or antisense strand of an siRNA, including deletion of a nucleotide or nucleotide pair at position 2, 3, 4, 5, 6, 7, or 8 such that a bulge is generated in the off-target transcript when it anneals with the sense and/or antisense strand of the siRNA; (4) nucleotide or nucleotide pair alterations at position 2, 3, 4, 5, 6, 7, or 8 of the sense and/or antisense strand of an siRNA, including insertions of a



nucleotide or nucleotide pair at position 2, such that a bulge is generated in the sense or antisense strand of the siRNA when it anneals with the off-target mRNA message, or (5) nucleotide or nucleotide pair alterations at position 2, 3, 4, 5, 6, 7, or 8 of the sense and/or antisense strand of an siRNA, including the presence of abasic  
5 nucleotides, or nucleotides with modifications at position C3 of the sugar ring, can be used to eliminate, minimize, or alter off-target effects in critical instances. The above-described mismatches can be used in conjunction with any of the embodiments described herein to reduce off-target effects.

10 That said, preferably, the siRNA of the first embodiment comprises from 18 – 24 base pairs, exclusive of overhangs. These molecules are not processed (or processed poorly) by the Type III RNase, Dicer, and for this reason, the pattern of modifications are preserved within the cell. Preferably, the sense strand and antisense strand are at least substantially complementary over the range of base pairs, and more  
15 preferably 100 % complementary over this range. Preferably, the polynucleotide is RNA.

The siRNA of the first embodiment may also contain overhangs of 1-6 nucleotides at either the 5' or 3' end of either the sense strand and/or the antisense  
20 strand. However, preferably if there are any overhangs, they are on the 3' end of the sense strand and/or the antisense strand. Additionally, preferably any overhangs are six or fewer bases in length, more preferably two or fewer bases in length. Most preferably, there are either no overhangs, or overhangs of two bases on one or both of the sense strand and antisense strand at the 3' end. Because overhanging nucleotides  
25 are frequently removed by one or more intracellular enzymatic processes or events, thus leaving an unphosphorylated 5'-nucleotide, it is preferable not to have overhangs on the 5' end of the antisense strand. In addition, overhangs can contain one or more stabilizing modifications, such as halogen modification of the 2' position or internucleotide modifications such as phosphorothioate, phosphorodithioate, or  
30 methylphosphonate modifications.

In further reference to the first embodiment, the phosphorylation of the first 5' terminal antisense nucleotide refers to the presence of one or more phosphate groups

attached to the 5' carbon of the sugar moiety of the nucleotide. Preferably there is only one phosphate group.

According to the present embodiment, the modification of the first and second  
5 5' sense nucleotides and the second 5' antisense nucleotides are a 2'-O-alkyl group.  
Preferably the modification is selected from the group consisting of 2'-O-methyl, 2'-  
O-ethyl, 2'-O-propyl, 2'-O-isopropyl, 2'-O-butyl, 2'-O-isobutyl, 2'-O-ethyl-O-methyl  
(-OCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>), and 2'-O-ethyl-OH (-OCH<sub>2</sub>CH<sub>2</sub>OH). Most preferably, the 2'-O-  
alkyl modification is a 2'-O-methyl moiety. Further, there is no requirement that the  
10 modification be the same on each of the first 5' sense nucleotide, the second 5' sense  
nucleotide, or the second antisense nucleotide. However, as a matter of practicality  
with respect to synthesizing the molecules of the present invention, it may be  
desirable to use the same modification throughout.

15 Alternatively, the molecule can have a sense strand where in the sense strand  
comprises a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises  
a first 2'-O-alkyl modification, and a second 5' sense nucleotide, wherein said second  
5' sense nucleotide comprises a second 2'-O-alkyl modification; and all Cs and Us  
(other than any of the aforementioned positions) are modified with a 2'-O-alkyl  
20 modification; and an antisense strand, wherein said antisense strand comprises a first  
5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated,  
and a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide  
comprises a third 2'-O-alkyl modification, and all Cs and Us (other than if present at  
the second 5' antisense nucleotide) are modified with a 2' F. Furthermore, these  
25 molecules can comprise a 2 nucleotide overhang on the 3' end of either or both  
strands and said overhang can further comprise a stabilized internucleotide linkage  
between: (1) the two nucleotides of the overhang; and (2) the penultimate nucleotide  
of the overhang and the final nucleotide of the duplexed region, comprises a  
phosphorothioate, a phosphorodithioate, or methylphosphonate linkage.

30 Alternatively, the molecule can contain a sense strand wherein the sense strand  
comprises a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises  
a 5' deoxy nucleotide; and an antisense strand, wherein said antisense strand  
comprises a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is

phosphorylated at the 5' carbon, and a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a 2'-O-alkyl modification. For a strand to participate in gene silencing by the RNAi pathway, the 5' end of that strand must be phosphorylated at the 5' carbon position. The presence of a 5' deoxynucleotide on the 5' end removes the functional group (-OH) from that strand, thus eliminating the ability of resident kinases to add a phosphate at this position. Without a phosphate group at the 5' terminus, the ability of this strand to be involved in RISC-mediated on- and off-target silencing is reduced.

While the invention identifies 2'-O-alkyl groups at the above-referenced positions, the inventors recognize that other chemical modification groups at similar or identical positions can also be used to minimize off-target effects. For example, the 2' modified nucleotide can be a 2' halogen modified nucleotide, a 2' amine modified nucleotide, and a 2' alkyl modified nucleotide if such modifications are included under conditions that minimize off-target effects. Where the modification is a halogen, the halogen is preferably fluorine. Where the 2' modified nucleotide is a 2' amine modified nucleotide, the amine is preferably -NH<sub>2</sub>. Where the 2' modified nucleotide is a 2'-alkyl modification, preferably the modification is selected from the group consisting of methyl, ethyl, propyl, isopropyl, butyl, or isobutyl moiety. Most preferably, a 2' methyl modification, wherein the carbon of the methyl moiety is attached directly to the 2' carbon of the sugar moiety, is used.

As stated above, the modification pattern described in the first embodiment is applicable for molecules that are not processed by Dicer. Yet in some instances, longer duplexes (*e.g.*, 25-30 basepairs) are preferred. These molecules are potential substrates for Dicer and for that reason, shifts in the pattern of modification and distinct patterns of overhang length and position must be incorporated into duplex design to insure that the entire complement of modifications described in the first embodiment are present in the final duplex, post-Dicer processing. In one non-limiting example, the structure of the duplex termini are engineered to guarantee the final product carries the necessary modifications in the appropriate positions. The side in which Dicer enters a duplex can be biased by the presence or absence of overhangs. Furthermore, the position of Dicer cleavage can be manipulated by varying the length of the 3' overhang. Thus, for example, one preferred design for

molecules that are 26 basepairs or longer includes: (1) a 1, 2, or 3 nucleotide overhang on the 3' end of the sense strand, and no overhangs on the opposite end of the duplex; (2) a 2'-O-alkyl modification on the second antisense nucleotide (counting from the 5' end of the strand); (3) phosphorylation of the 5' carbon of the first antisense nucleotide; and (4) paired 2'-O-alkyl modifications on sense strand nucleotides 21 and 22, 22 and 23, or 23 and 24 (counting from the 3' end of the sense strand, overhang included). Addition of the 3' sense strand overhang biases the side in which Dicer enters the duplex (*i.e.*, Dicer will preferably enter the side of the molecule that contains the overhang rather than the blunt end of the molecule). This ensures the preservation/retention of both antisense modifications (*i.e.*, the 2'-O-alkyl modification on the second antisense nucleotide, and the 5' phosphate group on the first antisense nucleotide) in the final, post-Dicer processing, siRNA. In addition, the positions of the sense strand modifications ensure that following Dicer processing, these modifications will be present on the terminal sense nucleotides (sense nucleotides 1 and 2) of the final, processed siRNA (see **Figure 1**).

According to a second embodiment, the present invention is directed to a unimolecular siRNA capable of forming a hairpin siRNA, said unimolecular siRNA comprising:

- a. a sense strand, comprising a sense region that comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
  - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense strand, comprising an antisense region that comprises
  - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated on the 5' carbon, and
  - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification, and

a loop region, wherein said loop region is located between said sense region and said antisense region and wherein said sense strand and said antisense strand are capable of forming a duplex of 18- 24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5'

sense nucleotide is the 5' most nucleotide of the duplex region, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand of the duplex region and said second 5' antisense nucleotide is immediately  
5 adjacent to and downstream of the first 5' antisense nucleotide.

According to this embodiment, the range of modifications is the same as those for the first embodiment. However, because the polynucleotide is unimolecular and is capable of forming a hairpin, and not two separate strands, there is one contiguous  
10 molecule that comprises both a sense region and an antisense region. Preferably, the sense region and the antisense region are at least substantially complementary, more preferably 100% complementary. Preferably the sense region and the antisense region comprise 19 – 35 base pairs, more preferably from 24-35 base pairs, and most preferably from 26 – 31 base pairs. Preferably, the entire length of the unimolecular  
15 siRNA contains fewer than 100 bases, more preferably fewer than 85 bases. Preferably the nucleotide is RNA.

Hairpins comprise two major components including a stem (which is a double stranded region pairing the antisense strand and the sense strand) and a loop.  
20 Optionally, an overhang sequence can be added to the 5', 3' or both ends of the molecule. Preferably, when an overhang is present, it is associated with the 3' end of the molecule. When designing a unimolecular siRNA, the hairpin can be designed as a left-handed hairpin (*e.g.*, 5'-AS-Loop-S) or a right-handed hairpin (*e.g.*, 5'-S-Loop-AS). Preferably, the hairpin is a left-handed hairpin. This construction is desirable  
25 because it is easier to phosphorylate the terminal antisense nucleotide. As was the case with double stranded siRNA, the length of the stem of the molecule determines whether or not it will be processed by Dicer. As hairpins containing longer stem structures are substrates for Dicer, the position of the chemical modifications must be adjusted to ensure that the final, post-Dicer processing product contains the  
30 modifications in the desired positions. Preferably, the post-Dicer processed molecule contains all of the modifications described in the first embodiment. In one non-limiting example, preferred hairpin designs for unimolecular molecules that have stems that are longer than 24 bases include the following properties: (1) a left-handed hairpin design, (2) a 1-3 nucleotide 3' overhang, (3) a phosphate group on the 5'

carbon of the 5' most nucleotide, (4) a 2'-O-alkyl (preferably an O-methyl) modification of the second antisense nucleotide, and (5) paired 2'-O-alkyl modifications on sense strand nucleotides 21 and 22, 22 and 23, or 23 and 24 (counting from the 3' end of the sense strand, overhang included). Addition of the 3' sense strand overhang enhances the ability of Dicer to enter the hairpin on the 5' AS end of the molecule. In addition, the positions of the sense strand modifications ensure that following Dicer processing, these modifications will be present on the terminal sense nucleotides (sense nucleotides 1 and 2 that have complementary bases on the antisense strand) of the final, processed siRNA (see **Figure 2**).

The hairpin may comprise a loop structure, which preferably comprises from four to ten bases. The bases of the loop can be modified. Alternatively, the loop can comprise non-nucleotide components such as those described in U.S. Patent Application No. 10/635108, published on March 25, 2004, as US 2004/0058886 A1. Preferable sequences of the loop structure include, for example, 5'-UUCG-3' (**SEQ. ID NO. 18**), 5'-UUUGUGUAG-3' (**SEQ. ID NO. 19**), 5'-CUUCCUGUCA-3' (**SEQ. ID NO. 20**), 5'-AUAUGUG-3' (**SEQ. ID NO. 21**), or any other loop identified in prior or pre-miRNA.

As described previously, the unimolecular siRNA of the present invention may ultimately be processed by cellular machinery such that they are converted into two separate strands. Further, these unimolecular siRNA may be introduced into the cell with less than all modifications, and modified in the cell itself through the use of natural processes or processing molecules that have been introduced (*e.g.*, phosphorylation in the cell). However, preferably the siRNA is introduced with all modifications already present. (Similarly, the strands of the first embodiment are preferably introduced into the cell with all modifications, though the antisense strand could, *e.g.*, be modified after introduction.) If a hairpin is processed by Dicer, the resulting hairpin should retain the modifications of the various embodiments described herein.

The above-described modifications should not be construed to suggest that no other moieties may be modified in addition to the nucleotides described, that also contribute to minimizing off-target effects or enhance other properties of the siRNA

(such as improved stability or functionality). Other types of modifications are permissible so long as they do not unacceptably increase off-target effects. In certain embodiments, such additional modifications can be added to one, two, three, or more consecutive nucleotides or every-other nucleotide of the sense strand. Alternatively, additional modifications can be confined to specific positions that have been identified as being key to sense strand entrance and/or use by RISC. As mentioned previously, further, additional modifications, such as 2'-O-alkyl groups (or other 2' modifications) can be added to one or more, preferably all, pyrimidines (*e.g.*, C and/or U nucleotides) of the sense strand and/or 2' F modifications (or other halogen modifications) can be added to one or more, preferably all pyrimidines (*e.g.*, C and/or U nucleotides) of the antisense strand other than to nucleotides that are otherwise modified as specified above. Modifications such as 2' F or 2'-O-alkyl of some or all of the Cs and Us of the antisense and/or sense strand (respectively) and other of the nucleotides specified above can greatly enhance the stability of the siRNA/shRNA molecules carrying the modifications described in embodiments 1 and 2, without appreciably altering target specific silencing.

Further, if a label is used in conjunction with the invention, these agents can be useful as tracking agents, which would assist in detection of transfection, as well as detection of where in the cell the molecule is present. Examples of commonly used labels include, but are not limited to, a fluorescent label, a radioactive label or a mass label.

Additionally stabilization modifications that are addressed to the phosphate backbone may also be included in the siRNAs for some applications of the present invention. For example, at least one phosphorothioate and/or methylphosphonate may be substituted for the phosphate group at some or all 3' positions of any or all pyrimidines in the sense and/or antisense strands of the oligonucleotide backbone, as well as in any overhangs, loop structures or stem structures that may be present. Phosphorothioate (and methylphosphonate) analogues arise from modification of the phosphate groups in the oligonucleotide backbone. In the phosphorothioate, the phosphate O<sup>-</sup> is replaced by a sulfur atom. In methylphosphonates, the oxygen is replaced with a methyl group. Additionally, phosphorothioate 3' modifications may be used instead of and independent of 2' fluoro modifications to increase stability of

an siRNA molecule. These modifications may be used in combination with the other modifications disclosed herein, or independent of those modifications in siRNA applications.

5           In other embodiments there can still be additional modifications such as those described as follows: (1) an antisense strand containing a 2' modification (preferably a 2'-O-alkyl modification) on the second antisense nucleotide, plus a phosphate group on carbon 5 of the first antisense nucleotide; plus *either* (2) positions 1, 2, and 3 of the sense strand (or sense region in an shRNA), counting from the 5' end of that strand  
10       are modified with 2' modifying groups (preferably a 2'-O-alkyl modification), *or* (3) the first sense nucleotide counting from the 5' end of that strand is a 5' deoxynucleotide. Either combination of modifications may be beneficial in substantially reducing both sense and antisense strand induced off-target effects.

15           In another embodiment the molecule comprises the following modifications: (1) the second terminal antisense nucleotide contains a 2' -O-alkyl modification; and (2) the first terminal antisense nucleotide contains a phosphate group on the 5' carbon; and (3) the 5' carbon of the first terminal nucleotide of the sense strand comprises any group known in the art that is suitable for blocking the hydroxyl group  
20       from accepting or being converted to a phosphate group. Preferably, the 5' terminal nucleotide blocking group comprises a 5'-O-alkyl, a 5' amine blocking group, or 5' azide blocking group.

          In other embodiments, any of the compositions of the present invention can  
25       further comprise a 3' cap. The 3' cap can be, for example, an inverted deoxythymidine.

          In other embodiments of the present invention, any of the compositions can comprise a conjugate. The conjugate can be selected from the group consisting of  
30       amino acids, peptides, polypeptides, proteins, sugars, carbohydrates, lipids, polymers, nucleotides, polynucleotides, and combinations thereof. The conjugate can be, for example, cholesterol or PEG. The conjugate can further comprise a label, such as, for example, a fluorescent label. The fluorescent label can be selected from the group



consisting of TAMRA, BODIPY, Cy3, Cy5, fluorescein, and Dabsyl. Alternatively, the fluorescent label can be any fluorescent label known in the art.

5 In other embodiments, the compositions of the present invention can comprise at least one 2'-orthoester modification, wherein the 2'-orthoester modification is preferably a 2'-bis(hydroxy ethyl) orthoester modification.

10 The duplex or duplex region can comprise one or more mismatches. For example, a duplex region can have one or more mismatches at any one or combination of positions 2 to 8 of, for example, the antisense strand. Nevertheless, the duplex region is considered in this case to include one or more mismatches where one or more mismatches can be counted among the 18-24 nucleotide basepairs that are at least 80% complementary. That the duplex region can comprise at least one mismatch can be included in any of the embodiments described herein.

15

The above described modifications of the present invention may be combined with siRNA that contains sequences that were selected at random, or according to any rational design selection procedure, for example, the rational design algorithm described in U.S. patent Application Serial No. 10/714,333, filed on November 14, 2003, entitled "Functional and Hyperfunctional siRNA"; in international patent application number PCT/US2003/036787, published on June 3, 2004 as WO 2004/045543 A2, entitled "Functional and Hyperfunctional siRNA; and in U.S. Patent Application Serial No. 10/940892, filed on September 14, 2004, entitled "Methods and Compositions for Selecting siRNA of Improved Functionality." Additionally, it may be desirable to select sequences in whole or in part based on internal thermal stability, which may facilitate processing by cellular machinery.

20 It should be noted that the modifications of the first and second embodiment of the present invention may have different effects depending on the functionality of the siRNA that are employed. Thus, in highly functional siRNA, the modifications of the present invention may cause a molecule to lose a certain amount of functionality, but would nonetheless be desirable because off-target effects are reduced. By contrast when moderately or poorly functional siRNA are used, there is very little functionality decrease and in some cases, functionality can increase.

30

A variety of approaches can be used to identify both the type of molecule and the key position needed to eliminate sense and/or antisense strand off-targeting effects. In one non-limiting example, a modification-function walk is performed. In this procedure, a single type of modification is added to one or more nucleotides across the sense and/or antisense strand. Subsequently, modified and unmodified molecules are tested for: (1) functionality; and (2) off-targeting effects, by one of several methods. Thus, for example, 2'-O-Me groups can be added to positions 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, 17 and 18, or 18 and 19 of either the sense and/or antisense strand and tested for functionality (*e.g.*, by measuring the ability of these molecules to silence specific targets) and off-target effects. If key positions are identified that eliminate some or all off-targeting, but result in a loss of duplex functionality, then a second round of modification walks, whereby additional chemical groups (*e.g.*, 5' phosphate on the 5' end of the antisense strand), mismatches, or bulges that are suspected to increase duplex functionality, can be added to molecules that already contain the modification that eliminates off-targeting.

In order to determine what modifications are permissible, several non-limiting assays can be performed to identify modifications that limit off-target effects. In one non-limiting example, the sense or antisense strand (carrying the modification being tested) can be labeled with one of many labeled nucleotides. Subsequently, a binding assay can be performed whereby the affinity of RISC for, *e.g.*, the modified sense strand can be compared with that of the unmodified form. Alternatively, siRNA containing various modifications can be transfected into cells by a variety of methodologies and cultures can subsequently be assessed by microarray analysis to determine whether the modifications alter the number and pattern of off-targeted genes.

According to a third embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing a modified siRNA containing the modifications described in embodiment 1, to a target nucleic acid, or a cell or organism that is either expressing the target nucleic acid or capable of expressing the target nucleic acid.

According to another embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing a unimolecular siRNA containing the modifications described in second embodiment to  
5 a target nucleic acid, or a cell or organism that is either expressing the target nucleic acid or capable of expressing the target nucleic acid.

In various embodiments, a 3' overhang of 1 to 6 bases on at least one of the sense strand and the antisense strand can be present. The overhang can be present  
10 with any of the embodiments described herein, unless otherwise specified or implicit from the context.

According to a fifth embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing two or more (*i.e.* a  
15 pool of) siRNA to a target nucleic acid or to a cell that is expressing or is capable of expressing said target nucleic acid, wherein said siRNAs comprise an antisense strand and a sense strand, wherein:

- a. a sense strand, wherein said sense strand comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense  
20 nucleotide comprises a first 2'-O-alkyl modification, and
  - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense strand, wherein said antisense strand comprises
  - i. a first 5' antisense nucleotide, wherein said first 5'  
25 antisense nucleotide is phosphorylated, and
  - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification,

30 wherein said sense strand and said antisense strand are capable of forming a duplex of 18 –24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense

nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

5           According to a sixth embodiment, the present invention is directed to a method for minimizing off-target effects, said method comprising exposing two or more (*i.e.* a pool of) unimolecular siRNAs (that are capable of forming a hairpin) to a target nucleic acid or to a cell that is expressing or is capable of expressing said target nucleic acid, wherein said unimolecular siRNA comprises an antisense region and a  
10   sense region, wherein:

          a. said sense region comprises

- i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
- ii. a second 5' sense nucleotide, wherein said second 5' sense  
15       nucleotide comprises a second 2'-O-alkyl modification; and

          b. said antisense region comprises

- i. i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
- ii. a second 5' antisense nucleotide, wherein said second  
20       5'antisense nucleotide comprises a third 2'-O-alkyl modification; and

          c. a loop region, wherein said loop region is located between said sense region and said antisense region.

25   wherein said sense region and said antisense region are capable of forming a duplex of 18-24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense region, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first 5' antisense  
30   nucleotide is the 5' most nucleotide of the antisense region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

In another embodiment, the invention comprises a kit, comprising at least two siRNA, wherein the at least two siRNA comprise a first siRNA and a second siRNA, and wherein each of the first siRNA and the second siRNA comprises:

- i. a sense strand, wherein said sense strand comprises
    - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and
    - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
  - ii. an antisense strand, wherein said antisense strand comprises
    - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
    - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;
- wherein said sense strand and said antisense strand are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

In some embodiments the kit comprises siRNAs wherein the first siRNA and the second siRNA each comprises a sequence that is at least substantially complementary to a region in a target mRNA. In some embodiments the kit comprises siRNAs wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary. In some embodiments the kit comprises siRNAs such that the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.

In another embodiment, the invention comprises a kit, comprising at least two unimolecular siRNA, wherein the at least two siRNA comprise a first siRNA and a second siRNA, and wherein each of the first siRNA and the second siRNA comprises:

a. a sense region, wherein said sense region comprises

- 5           i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and
- ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and

b. an antisense region, wherein said antisense region comprises

- 10          i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
- ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;

wherein said sense region and said antisense region are capable of forming a  
15   duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense region, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the  
20   antisense region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

The kit can comprise siRNAs such that the first siRNA and the second siRNA each comprises a sequence that is at least substantially complementary to a region in a  
25   target mRNA. In some embodiments, the kit comprises siRNAs such that the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary. In some embodiments, the kit comprises siRNAs such that the region of the target mRNA to which the sequence of  
30   the first siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.

In another embodiment, the invention comprises a method for minimizing off-target effects in RNAi, said method comprising:

exposing at least two unimolecular siRNA to a target nucleic acid or to a cell, wherein the at least two unimolecular siRNA comprise a first unimolecular siRNA  
5 and a second unimolecular siRNA, wherein each of the first unimolecular siRNA and the second unimolecular siRNA comprises

- a. a sense region, wherein said sense region comprises
  - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and
  - 10 ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense region, wherein said antisense region comprises
  - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
  - 15 ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;

wherein said sense region and said antisense region are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5'  
20 sense nucleotide is the 5' most nucleotide of the sense region, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

25 In some embodiments, the method comprises using siRNAs wherein the first unimolecular siRNA and the second unimolecular siRNA each comprises a sequence that is at least substantially complementary to a region in a target mRNA. In some embodiments, the method comprises using siRNAs such that the region of the target  
30 mRNA to which the sequence of the first unimolecular siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second unimolecular siRNA is at least substantially complementary. In some embodiments, the method comprises using siRNAs such that the region of the target mRNA to which the sequence of the first unimolecular siRNA is at least substantially

complementary does not overlap the region of the target mRNA to which the sequence of the second unimolecular siRNA is at least substantially complementary.

5 In another embodiment, the invention comprises a method for minimizing off-target effects in RNAi, said method comprising:

exposing at least two siRNA to a target nucleic acid or to a cell, wherein the at least two siRNA comprise a first siRNA and a second siRNA, wherein each of the first siRNA and the second siRNA comprises

- a. a sense strand, wherein said sense strand comprises
  - 10 i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and
  - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
- b. an antisense strand, wherein said antisense strand comprises
  - 15 i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
  - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;

wherein said sense strand and said antisense strand are capable of forming a duplex of  
20 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second  
25 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

In some embodiments, the method comprises using siRNAs such that the first siRNA and the second siRNA each comprises a sequence that is at least substantially  
30 complementary to a region in a target mRNA. In some embodiments, the method comprises using siRNAs such that the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary. In some embodiments, the method comprises using



siRNAs such that the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.

5

Because the ability of the dsRNA of the present invention to retain functionality and exhibit improved specificity is not dependent on the sequence of the bases, the cell type, or the species into which it is introduced, the present invention is applicable across a broad range of organisms, including but not limited to plants, animals, protozoa, bacteria, viruses and fungi. The present invention is particularly advantageous for use in mammals such as cattle, horse, goats, pigs, sheep, canines, rodents such as hamsters, mice, and rats, and primates such as, gorillas, chimpanzees, and humans.

15 The present invention may be used advantageously with diverse cell types, including but not limited to primary cells, germ cell lines and somatic cells. The cells may be, for example, stem cells or differentiated cells. For example, the cell types may be embryonic cells, oocytes, sperm cells, adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes and cells of the endocrine or exocrine glands.

25 The present invention is applicable for use for employing RNA interference (and/or using as a control) directed against a broad range of genes, including but not limited to the 45,000 genes of a human genome, such as those implicated in diseases such as diabetes, Alzheimer's and cancer, as well as all genes in the genomes of the humans, mice, hamsters, chimpanzees, goats, sheep, horses, camels, pigs, dogs, cats, nematodes (*e.g.*, *C. elegans*), flies (*e.g.*, *D. melanogaster*), and other vertebrates and invertebrates.

30

The siRNAs of the present invention may be administered to a cell by any method that is now known or that comes to be known and that from reading this disclosure, one skilled in the art would conclude would be useful with the present

invention. For example, the siRNAs may be passively delivered to cells. Passive uptake of modified siRNAs can be modulated, for example, by the presence of a conjugate such as a polyethylene glycol moiety or a cholesterol moiety at the 5' terminal of the sense strand and/or, in appropriate circumstances, a pharmaceutically acceptable carrier.

Other methods for delivery include, but are not limited to, transfection techniques employing DEAE-Dextran, calcium phosphate, cationic lipids/liposomes, microinjection, electroporation, immunoporation, and coupling of the siRNAs to specific conjugates or ligands such as antibodies, peptides, antigens, or receptors.

Preferably, the siRNAs comprise duplexes when they are administered.

Further, the method of assessing the level of gene silencing is not limited. Thus, the silencing ability of any given siRNA can be studied by one of any number of art tested procedures including but not limited to Northern analysis, Western Analysis, RT PCR, expression profiling, and others.

The polynucleotides of the present invention may be synthesized by any method that is now known or that comes to be known and that from reading this disclosure a person of ordinary skill in the art would appreciate would be useful to synthesize the molecules of the present invention. siRNA duplexes containing the specified modifications may be chemically synthesized using compositions of matter and methods described in Scaringe, S.A. (2000) "Advanced 5'-silyl-2'-orthoester approach to RNA oligonucleotide synthesis," *Methods Enzymol.* **317**, 3-18; Scaringe, S.A. (2001) "RNA oligonucleotide synthesis via 5'-silyl-2'-orthoester chemistry," *Methods* **23**, 206-217; Scaringe, S. and Caruthers, M.H. (1999) U.S. Patent No. 5,889,136; Scaringe, S. and Caruthers, M.H. (1999) U.S. Patent No. 6,008,400; Scaringe, S. (2000) U.S. Patent No. 6,111,086; Scaringe, S. (2003) U.S. Patent No. 6,590,093. The synthesis method utilizes nucleoside base-protected 5'-O-silyl-2'-O-orthoester-3'-O-phosphoramidites to assemble the desired unmodified siRNA sequence on a solid support in the 3' to 5' direction. Briefly, synthesis of the required phosphoramidites begins from standard base-protected ribonucleosides (uridine, N<sup>4</sup>-acetylcytidine, N<sup>2</sup>-isobutyrylguanosine and N<sup>6</sup>-isobutyryladenosine). Introduction of

the 5'-O-silyl and 2'-O-orthoester protecting groups, as well as the reactive 3'-O-phosphoramidite moiety is then accomplished in five steps, including:

1. Simultaneous transient blocking of the 5'- and 3'-hydroxyl groups of the nucleoside sugar with Markiewicz reagent (1,3-dichloro-1,1,3,3,-tetraisopropylidisiloxane [TIPS-Cl<sub>2</sub>]) in pyridine solution {Markiewicz, W.T. (1979) "Tetraisopropylidisiloxane-1,3-diyl, a Group for Simultaneous Protection of 3'- and 5'-Hydroxy Functions of Nucleosides," *J. Chem. Research(S)*, 24-25}, followed by chromatographic purification;
2. Regiospecific conversion of the 2'-hydroxyl of the TIPS-nucleoside sugar to the *bis*(acetoxylethyl)orthoester [ACE derivative] using tris(acetoxylethyl)-orthoformate in dichloromethane with pyridinium p-toluenesulfonate as catalyst, followed by chromatographic purification;
3. Liberation of the 5'- and 3'-hydroxyl groups of the nucleoside sugar by specific removal of the TIPS-protecting group using hydrogen fluoride and N,N,N',N'-tetramethylethylene diamine in acetonitrile, followed by chromatographic purification;
4. Protection of the 5'-hydroxyl as a 5'-O-silyl ether using benzhydroxy-*bis*(trimethylsilyloxy)silyl chloride [BzH-Cl] in dichloromethane, followed by chromatographic purification; and
5. Conversion to the 3'-O-phosphoramidite derivative using *bis*(N,N-diisopropylamino)methoxyphosphine and 5-ethylthio-1H-tetrazole in dichloromethane/acetonitrile, followed by chromatographic purification.

The phosphoramidite derivatives are typically thick, colorless to pale yellow syrups. For compatibility with automated RNA synthesis instrumentation, each of the products is dissolved in a pre-determined volume of anhydrous acetonitrile, and this solution is aliquoted into the appropriate number of serum vials to yield a 1.0-mmol quantity of phosphoramidite in each vial. The vials are then placed in a suitable vacuum desiccator and the solvent removed under high vacuum overnight. The

atmosphere is then replaced with dry argon, the vials are capped with rubber septa, and the packaged phosphoramidites are stored at  $-20^{\circ}\text{C}$  until needed. Each phosphoramidite is dissolved in sufficient anhydrous acetonitrile to give the desired concentration prior to installation on the synthesis instrument.

5

The synthesis of the desired oligoribonucleotide is carried out using automated synthesis instrumentation. It begins with the 3'-terminal nucleoside covalently bound via its 3'-hydroxyl to a solid beaded polystyrene support through a cleavable linkage. The appropriate quantity of support for the desired synthesis scale is measured into a reaction cartridge, which is then affixed to synthesis instrument. The bound nucleoside is protected with a 5'-O-dimethoxytrityl moiety, which is removed with anhydrous acid (3% [v/v] dichloroacetic acid in dichloromethane) in order to free the 5'-hydroxyl for chain assembly.

Subsequent nucleosides in the sequence to be assembled are sequentially added to the growing chain on the solid support using a four-step cycle, consisting of the following general reactions:

1. Coupling: the appropriate phosphoramidite is activated with 5-ethylthio-1H-tetrazole and allowed to react with the free 5'-hydroxyl of the support bound nucleoside or oligonucleotide. Optimization of the concentrations and molar excesses of these two reagents, as well as of the reaction time, results in coupling yields generally in excess of 98% per cycle.
2. Oxidation: the internucleotide linkage formed in the coupling step leaves the phosphorous atom in its P(III) [phosphite] oxidation state. The biologically-relevant oxidation state is P(V) [phosphate]. The phosphorous is therefore oxidized from P(III) to P(V) using a solution of *tert*-butylhydroperoxide in toluene.
3. Capping: the small quantity of residual un-reacted 5'-hydroxyl groups must be blocked from participation in subsequent coupling cycles in order to prevent the formation of deletion-containing sequences. This is accomplished by treating the support with a large excess of acetic anhydride and 1-

methylimidazole in acetonitrile, which efficiently blocks residual 5'-hydroxyl groups as acetate esters.

4. De-silylation: the silyl-protected 5'-hydroxyl must be deprotected prior to the next coupling reaction. This is accomplished through treatment with triethylamine trihydrogen fluoride in N,N-dimethylformamide, which rapidly and specifically liberates the 5'-hydroxyl without concomitant removal of other protecting groups (2'-O-ACE, N-acyl base-protecting groups, or phosphate methyl).

It should be noted that in between the above four reaction steps are several washes with acetonitrile, which are employed to remove the excess of reagents and solvents prior to the next reaction step. The above cycle is repeated the necessary number of times until the unmodified portion of the oligoribonucleotide has been assembled. The above synthesis method is only exemplary and should not be construed as limited the means by which the molecules may be made. Any method that is now known or that comes to be known for synthesizing siRNA and that from reading this disclosure one skilled in the art would conclude would be useful in connection with the present invention may be employed.

The siRNA duplexes of certain embodiments include two modified nucleosides (*e.g.*, 2'-O-methyl derivatives) at the 5'-end of each strand. The 5'-O-silyl-2'-O-methyl-3'-O-phosphoramidite derivatives required for the introduction of these modified nucleosides are prepared using procedures similar to those described previously (steps 4 and 5 above), starting from base-protected 2'-O-methyl nucleosides (2'-O-methyl-uridine, 2'-O-methyl-N<sup>4</sup>-acetylcytidine, 2'-O-methyl-N<sup>2</sup>-isobutrylguanosine and 2'-O-methyl-N<sup>6</sup>-isobutryladenosine). The absence of the 2'-hydroxyl in these modified nucleosides eliminates the need for ACE protection of these compounds. As such, introduction of the 5'-O-silyl and the reactive 3'-O-phosphoramidite moiety is accomplished in two steps, including:

1. Protection of the 5'-hydroxyl as a 5'-O-silyl ether using benzhydroxy-bis(trimethylsilyloxy)silyl chloride (BzH-Cl) in N,N-dimethylformamide, followed by chromatographic purification; and

2. Conversion to the 3'-O-phosphoramidite derivative using *bis*(N,N-diisopropylamino)methoxyphosphine and 5-ethylthio-1H-tetrazole in dichloromethane/acetonitrile, followed by chromatographic purification.

5           Post-purification packaging of the phosphoramidites is carried out using the procedures described previously for the standard nucleoside phosphoramidites. Similarly, the incorporation of the two 5'-O-silyl-2'-O-methyl nucleosides via their phosphoramidite derivatives is accomplished by twice applying the same four-step cycle described previously for the standard nucleoside phosphoramidites.

10           The siRNA duplexes of certain embodiments of this invention include a phosphate moiety at the 5'-end of the antisense strand. This phosphate is introduced chemically as the final coupling to the antisense sequence. The required phosphoramidite derivative (*bis*(cyanoethyl)-N,N-diisopropylamino phosphoramidite) is synthesized as follows in brief: phosphorous trichloride is treated one equivalent of N,N-diisopropylamine in anhydrous tetrahydrofuran in the presence of excess triethylamine. Then, two equivalents of 3-hydroxypropionitrile are added and allowed to react completely. Finally, the product is purified by chromatography. Post-purification packaging of the phosphoramidite is carried out using the procedures described previously for the standard nucleoside phosphoramidites. Similarly, the incorporation of the phosphoramidite at the 5'-end of the antisense strand is accomplished by applying the same four-step cycle described previously for the standard nucleoside phosphoramidites.

25           The modified, protected oligoribonucleotide remains linked to the solid support at the finish of chain assembly. A two-step rapid cleavage/deprotection procedure is used to remove the phosphate methyl protecting groups, cleave the oligoribonucleotide from the solid support, and remove the N-acyl base-protecting groups. It should be noted that this procedure also removes the cyanoethyl protecting groups from the 5'-phosphate on the antisense strand. Additionally, the procedure removes the acetyl functionalities from the ACE orthoester, converting the 2'-O-ACE protecting group into the *bis*(2-hydroxyethyl)orthoester. This new orthoester is significantly more labile to mild acid as well as more hydrophilic than the parent ACE group. The two-step procedure is briefly as follows:

1. The support-bound oligoribonucleotide is treated with a solution of disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate in N,N-dimethylformamide. This reagent rapidly and efficiently removes the methyl protecting groups from the internucleotide phosphate linkages without cleaving the oligoribonucleotide from the solid support. The support is then washed with water to remove excess dithiolate.
2. The oligoribonucleotide is cleaved from the solid support with 40% (w/v) aqueous methylamine at room temperature. The methylamine solution containing the crude oligoribonucleotide is then heated to 55°C to remove the protecting groups from the nucleoside bases. The crude orthoester-protected oligoribonucleotide is obtained following solvent removal *in vacuo*.

Removal of the 2'-orthoesters is the final step in the synthesis process. This is accomplished by treating the crude oligoribonucleotide with an aqueous solution of acetic acid and N,N,N',N'-tetramethyl ethylene diamine, pH 3.8, at 55°C for 35 minutes. The completely deprotected oligoribonucleotide is then desalted by ethanol precipitation and isolated by centrifugation.

In addition, incorporation of fluorescent labels at the 5'-terminus of a polynucleotide is a common and well-understood manipulation for those skilled in the art. In general, there are two methods that are employed to accomplish this incorporation, and the necessary materials are available from several commercial sources (*e.g.*, Glen Research Inc., Sterling, Virginia, USA; Molecular Probes Inc., Eugene, Oregon, USA; TriLink BioTechnologies Inc., San Diego, California, USA; and others). The first method utilizes a fluorescent molecule that has been derivatized with a phosphoramidite moiety similar to the phosphoramidite derivatives of the nucleosides described previously. In such case, the fluorescent dye is appended to the support-bound polynucleotide in the final cycle of chain assembly. The fluorophore-modified polynucleotide is then cleaved from the solid support and deprotected using the standard procedures described above. This method has been termed "direct labeling." Alternatively, the second method utilizes a linker molecule derivatized

with a phosphoramidite moiety that contains a protected reactive functional group (e.g., amino, sulfhydryl, carbonyl, carboxyl, and others). This linker molecule is appended to the support-bound polynucleotide in the final cycle of chain assembly. The linker-modified polynucleotide is then cleaved from the solid support and  
5 deprotected using the standard procedures described above. The functional group on the linker is deprotected either during the standard deprotection procedure, or by utilizing a subsequent group-specific treatment. The crude linker-modified polynucleotide is then reacted with an appropriate fluorophore derivative that will result in formation of a covalent bond between a site on the fluorophore and the  
10 functional group of the linker. This method has been termed "indirect labeling."

Once synthesized, the polynucleotides of the present invention may immediately be used or be stored for future use. Preferably, the polynucleotides of the invention are stored as duplexes in a suitable buffer. Many buffers are known in  
15 the art suitable for storing siRNAs. For example, the buffer may be comprised of 100 mM KCl, 30 mM HEPES-pH 7.5, and 1mM MgCl<sub>2</sub>. Preferably, the siRNAs of the present invention retain 30% to 100% of their activity when stored in such a buffer at 4°C for one year. More preferably, they retain 80% to 100% of their biological activity when stored in such a buffer at 4°C for one year. Alternatively, the  
20 compositions can be stored at -20°C in such a buffer for at least a year or more. Preferably, storage for a year or more at -20°C results in less than a 50% decrease in biological activity. More preferably, storage for a year or more at -20°C results in less than a 20% decrease in biological activity after a year or more. Most preferably, storage for a year or more at -20°C results in less than a 10% decrease in biological  
25 activity.

In order to ensure stability of the siRNA pools prior to usage, they may be retained in dried-down form at -20°C until they are ready for use. Prior to usage, they should be resuspended; however, once resuspended, for example, in the  
30 aforementioned buffer, they should be kept at -20°C until used. The aforementioned buffer, prior to use, may be stored at approximately 4°C or room temperature. Effective temperatures at which to conduct transfection are well known to persons skilled in the art, and include for example, room temperature.



In order to form the duplex siRNA from the component complementary strands, equal quantities of the sense strand and antisense strand are mixed. Since the oligonucleotides retain the 2'-orthoester protection at this point, they are treated with mild acid at 55°C, which treatment removes these protecting groups. The deprotected  
5 strands are annealed to form the duplex by allowing the deprotection solution to cool slowly to room temperature. Finally, the duplex is desalted by precipitating it with ethanol, and the purified duplex is dissolved in RNase-free water and quantified by ultraviolet spectroscopy. The quality of the duplexing process is assessed by native gel electrophoresis.

10

Applications for this new and novel technology are broad. For instance, it is possible that an individual may identify one or more siRNA directed against, *e.g.*, a therapeutically important target. Said molecule might provide excellent silencing of the target of interest, but simultaneously silence additional genes (off-targets) that  
15 have partial complementarity with the sense and/or antisense strand of the siRNA. Silencing of these secondary targets (off-targets) can induce undesirable effects (*e.g.*, cell death, cell proliferation, differentiation) and for this reason, it is advantageous to eliminate the off-target effects associated with the siRNA of interest.

20

Further, the siRNA of the present invention may be used in a diverse set of applications, including but not limited to basic research, drug discovery and development, diagnostics, and therapeutics. In research settings, the application can involve introduction of modified molecules into cells using either a reverse transfection or forward transfection protocol. For example, the present invention may  
25 be used to validate whether a gene product is a target for drug discovery or development. In this application, the mRNA that corresponds to a target nucleic acid sequence of interest is identified for targeted degradation. Inventive siRNAs that are specific for targeting the particular gene are introduced into a cell or organism, preferably in duplex form. The cell or organism is maintained under conditions  
30 allowing for the degradation of the targeted mRNA, resulting in decreased activity or expression of the gene. The extent of any decreased expression or activity of the gene is then measured, along with the effect of such decreased expression or activity, and a determination is made that if expression or activity is decreased, then the nucleic acid sequence of interest is an agent for drug discovery or development. In this manner,

phenotypically desirable effects can be associated with RNA interference of particular target nucleic acids of interest, and in appropriate cases toxicity and pharmacokinetic studies can be undertaken and therapeutic preparations developed.

5           The invention can be implemented for a wide range of applications associated with transfection of the modified molecules. In one non-limiting example, modifications of the invention are used to eliminate off-target effects resulting from transfection of siRNA in a reverse transfection format. For example, the modified siRNA are dried on a solid surface (*e.g.*, the bottom of a well in a 96, 384, or 1536  
10 well plate), solubilized by the addition of a carrier (*e.g.*, a lipid transfection reagent), followed by the addition of the cell type(s) of choice for transfection.

          In another application of the invention, siRNA that are modified with the modifications of the invention, or unmodified, are separately transfected into cells and  
15 run side-by-side to identify or distinguish between gene-knockdown induced phenotypic outcomes that are generated by target specific silencing and off-target silencing.

          In another application of using the modified siRNA of the invention, cells are  
20 transfected with pools of modified siRNA or modified individual siRNA that constitute the pools. In this way, a user is able to identify the most functional siRNA or combination of siRNAs against an individual target.

          In yet another application, siRNA carrying the modifications of the invention  
25 are directed against a particular family of genes (*e.g.*, kinases), genes associated with a particular pathway(s) (*e.g.*, cell cycle regulation), or entire genomes (*e.g.*, the human, rat, mouse, *C. elegans*, or *Drosophila* genome). Knockdown of each gene of the collection with siRNA carrying the modifications of the invention would enable researchers to assess quickly the contribution of each member of a family of genes, or  
30 each member of a pathway, or each gene in a genome, to a particular biological function or event without the risk that the phenotype is the result of an off-target effect. As one example of this sort of application, individuals who are interested in identifying one or more host (human) genes that contribute to the ability of, *e.g.*, the HIV virus to infect human cells, can plate siRNA directed against the entire human

genome in a RTF format. Following lipoplex formation, cells that are susceptible to HIV infection (*e.g.*, JC53 cells) are added to each well for transfection. After culturing the cells for a period of 24-48 hours, the cells in each well could be subjected to a lethal titer of the HIV virus. Following an appropriate incubation period necessary for infection, plates could be examined to identify which wells contain living cells. Wells that contain living cells (or a substantially larger number of living cells than controls) identify a host gene that is necessary for viral infection, replication, and/or release. In this way, one is able to identify host genes that play a role in pathogen infection with the risk that the observed phenotype is the result of off-target effects.

In yet another application, cells transfected with siRNA carrying the modifications of the invention are used to assess a particular gene's (target's) contribution to exclusion of a drug from cells. In one non-limiting example, cells are reverse transfected on RTF plates that contain siRNA(s) directed against all known members of the human genome, siRNA directed against a particular family of genes (*e.g.*, kinases), siRNA directed against genes of a particular pathway (*e.g.*, the ADME-tox pathways). Subsequently, cells are treated with a particular compound (*e.g.*, a potential therapeutic compound) and the ability of cells to, *e.g.*, retain, excrete, metabolize, or adsorb that compound can be measured and compared with untreated cells. In this way, a researcher can identify one or more host genes that play a role in the pharmacokinetics of the compound with limited risk that the observed phenotype is the result of down-regulation of an off-target gene.

In yet another application, cells transfected with siRNA carrying the modifications of the invention are used to validate the target of one or more biologically relevant agents (*e.g.*, a drug). For instance, if a particular drug is believed to target a particular protein and induce a particular phenotype, the action of the drug can be validated by targeting its target protein with a gene-specific siRNA carrying the modifications of the invention. If the siRNA induces the same phenotype as the drug, then the target is validated. If the modified siRNA fails to induce the same phenotype, then these experiments would question the validity of the proposed protein as the drug target.

In yet another application, two or more siRNAs carrying the modifications of the invention and targeting two or more distinct targets can be used to identify and study synthetic lethal pairs.

5           In yet another application, siRNA carrying the modifications of the invention can be used to target transcripts containing single nucleotide polymorphisms (SNPs) to facilitate and assess the contribution of a particular SNP to a phenotype, a biological function, a disease state, or event.

10           In yet another application, siRNA carrying the modifications of the invention can be used to target a gene(s) whose knockdown is known to induce a particular disease state. In this way, it is possible to facilitate study of that particular disease without the risk of knocking down the expression of additional genes.

15           In all of the applications described above, the applications can be employed in such a way as to knock down one or multiple genes in a single well.

          The present invention may also be used in RNA interference applications that induce transient or permanent states of disease or disorder in an organism by, for  
20           example, attenuating the activity of a target nucleic acid of interest believed to be a cause or factor in the disease or disorder of interest. Increased activity of the target nucleic acid of interest may render the disease or disorder worse, or tend to ameliorate or to cure the disease or disorder of interest, as the case may be. Likewise, decreased activity of the target nucleic acid of interest may cause the disease or disorder, render  
25           it worse, or tend to ameliorate or cure it, as the case may be. Target nucleic acids of interest can comprise genomic or chromosomal nucleic acids or extrachromosomal nucleic acids, such as viral nucleic acids.

          Still further, the present invention may be used in RNA interference  
30           applications, such as diagnostics, prophylactics, and therapeutics including use of the compositions in the manufacture of a medicament in animals, preferably mammals, more preferably humans in the treatment of diseases, or over or under expression of a target. Preferably, the disease or disorder is one that arises from the malfunction of one or more proteins, the disease or disorder of which is related to the expression of

the gene product of the one or more proteins. For example, it is widely recognized that certain cancers of the human breast are related to the malfunction of a protein expressed from a gene commonly known as the "*bcl-2*" gene. A medicament can be manufactured in accordance with the compositions and teachings of the present

5 invention, employing one or more siRNAs directed against the *bcl-2* gene, and optionally combined with a pharmaceutically acceptable carrier, diluent and/or adjuvant, which medicament can be used for the treatment of breast cancer. Applicants have established the utility of the methods and compositions in cellular models. Methods of delivery of polynucleotides, such as siRNAs, to cells within  
10 animals, including humans, are well known in the art. Any delivery vehicle now known in the art, or that comes to be known, and has utility for introducing polynucleotides, such as siRNAs, to animals, including humans, is expected to be useful in the manufacture of a medicament in accordance with the present invention, so long as the delivery vehicle is not incompatible with any modifications that may be  
15 present within a composition made according to the present invention. A delivery vehicle that is not compatible with a composition made according to the present invention is one that reduces the efficacy of the composition by greater than 95% as measured against efficacy in cell culture.

20 Animal models exist for many, many disorders, including, for example, cancers, diseases of the vascular system, inborn errors or metabolism, and the like. It is within ordinary skill in the art to administer nucleic acids to animals in dosing regimens to arrive at an optimal dosing regimen for particular disease or disorder in an animal such as a mammal, for example, a mouse, rat or non-human primate. Once  
25 efficacy is established in the mammal by routine experimentation by one of ordinary skill, dosing regimens for the commencement of human trials can be arrived at based on data arrived at in such studies.

Dosages of medicaments manufactured in accordance with the present  
30 invention may vary from micrograms per kilogram to hundreds of milligrams per kilogram of a subject. As is known in the art, dosage will vary according to the mass of the mammal receiving the dose, the nature of the mammal receiving the dose, the severity of the disease or disorder, and the stability of the medicament in the serum of the subject, among other factors well known to persons of ordinary skill in the art.

For these applications, an organism suspected of having a disease or disorder that is amenable to modulation by manipulation of a particular target nucleic acid of interest is treated by administering siRNA. Results of the siRNA treatment may be ameliorative, palliative, prophylactic, and/or diagnostic of a particular disease or disorder. Preferably, the siRNA is administered in a pharmaceutically acceptable manner with a pharmaceutically acceptable carrier or diluent.

Therapeutic applications of the present invention can be performed with a variety of therapeutic compositions and methods of administration. Pharmaceutically acceptable carriers and diluents are known to persons skilled in the art. Methods of administration to cells and organisms are also known to persons skilled in the art. Dosing regimens, for example, are known to depend on the severity and degree of responsiveness of the disease or disorder to be treated, with a course of treatment spanning from days to months, or until the desired effect on the disorder or disease state is achieved. Chronic administration of siRNAs may be required for lasting desired effects with some diseases or disorders. Suitable dosing regimens can be determined by, for example, administering varying amounts of one or more siRNAs in a pharmaceutically acceptable carrier or diluent, by a pharmaceutically acceptable delivery route, and amount of drug accumulated in the body of the recipient organism can be determined at various times following administration. Similarly, the desired effect (for example, degree of suppression of expression of a gene product or gene activity) can be measured at various times following administration of the siRNA, and this data can be correlated with other pharmacokinetic data, such as body or organ accumulation. A person of ordinary skill in the art can determine optimum dosages, dosing regimens, and the like. A person of ordinary skill in the art may employ EC<sub>50</sub> data from *in vivo* and *in vitro* animal models as guides for human studies.

Still further, the present invention may be used in RNA interference applications, such as diagnostics, prophylactics, and therapeutics. For these applications, an organism suspected of having a disease or disorder that is amenable to modulation by manipulation of a particular target nucleic acid of interest is treated by administering siRNA. Results of the siRNA treatment may be ameliorative, palliative, prophylactic, and/or diagnostic of a particular disease or disorder.

Preferably, the siRNA is administered in a pharmaceutically acceptable manner with a pharmaceutically acceptable carrier and/or diluent.

Further, the siRNAs can be administered in a cream or ointment topically, an oral preparation such as a capsule or tablet or suspension or solution, and the like. The route of administration may be intravenous, intramuscular, dermal, subdermal, cutaneous, subcutaneous, intranasal, oral, rectal, by eye drops, by tissue implantation of a device that releases the siRNA at an advantageous location, such as near an organ or tissue or cell type harboring a target nucleic acid of interest.

Having described the invention with a degree of particularity, examples will now be provided. These examples are not intended to and should not be construed to limit the scope of the claims in any way. Although the invention may be more readily understood through reference to the following examples, they are provided by way of illustration and are not intended to limit the present invention unless specified.

## EXAMPLES

### EXAMPLE 1 SYNTHESIZING siRNAs

RNA oligonucleotides were synthesized using 2'-ACE chemistry (see **Figure 3**). The synthesis is preferably carried out as an automated process on an appropriate machine. Several such synthesizing machines are known to those of skill in the art. Each nucleotide is added sequentially (3'- to 5'-direction) to a solid support-bound oligonucleotide. Although polystyrene supports are preferred, any suitable support can be used. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support. The nucleotide precursor, an activated ribonucleotide such as a phosphoramidite or H-phosphonate, and an activator such as a tetrazole, for example, S-ethyl-tetrazole (although any other suitable activator can be used) are added (step i in **Figure 3**), coupling the second base onto the 5'-end of the first nucleoside. The support is washed and any unreacted 5'-hydroxyl groups are capped with an acetylating reagent such as, but not limited to, acetic anhydride or phenoxyacetic anhydride to yield unreactive 5'-acetyl moieties (step ii). The P(III) linkage is then oxidized to the more stable and ultimately desired P(V) linkage (step iii), using a

suitable oxidizing agent such as, for example, *t*-butyl hydroperoxide or iodine and water. At the end of the nucleotide addition cycle, the 5'-silyl group is cleaved with fluoride ion (step **iv**), for example, using triethylammonium fluoride or *t*-butyl ammonium fluoride. The cycle is repeated for each subsequent nucleotide. It should  
5 be emphasized that although **Figure 3** illustrates a phosphoramidite having a methyl protecting group, any other suitable group may be used to protect or replace the oxygen of the phosphoramidite moiety. For example, alkyl groups, cyanoethyl groups, or thio derivatives can be employed at this position. Further, the incoming activated nucleoside in step (i) can be a different kind of activated nucleoside, for  
10 example, an H-phosphonate, methyl phosphonamidite or a thiophosphoramidite. It should be noted that the initial, or 3', nucleoside attached to the support can have a different 5' protecting group such as a dimethoxytrityl group, rather than a silyl group. Cleavage of the dimethoxytrityl group requires acid hydrolysis, as employed in standard DNA synthesis chemistry. Thus, an acid such as dichloroacetic acid  
15 (DCA) or trichloroacetic acid (TCA) is employed for this step alone. Apart from the DCA cleavage step, the cycle is repeated as many times as necessary to synthesize the polynucleotide desired.

Following synthesis, the protecting groups on the phosphates, which are  
20 depicted as methyl groups in **Figure 3**, but need not be limited to methyl groups, are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (dithiolate) in DMF (dimethylformamide). The deprotection solution is washed from the solid support bound oligonucleotide using water. The support is then treated with 40% methylamine for 20 minutes at 55 °C. This releases  
25 the RNA oligonucleotides into solution, deprotects the exocyclic amines and removes the acetyl protection on the 2'-ACE groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2'-orthoester groups are the last protecting groups to be removed, if  
30 removal is desired. The structure of the 2'-ACE protected RNA immediately prior to 2'-deprotection is as represented in **Figure 4**.

For automated procedures, solid supports having the initial nucleoside are installed in the synthesizing instrument. The instrument will contain all the necessary



ancillary reagents and monomers needed for synthesis. Reagents are maintained under argon, since some monomers, if not maintained under an inert gas, can hydrolyze. The instrument is primed so as to fill all lines with reagent. A synthesis cycle is designed that defines the delivery of the reagents in the proper order according to the synthesis cycle, delivering reagents in a specified order. Once a cycle is defined, the amount of each reagent to be added is defined, the time between steps is defined, and washing steps are defined, synthesis is ready to proceed once the solid support having the initial nucleoside is added.

For the RNA analogs described herein, modification is achieved through three different general methods. The first, which is implemented for carbohydrate and base modifications, as well as for introduction of certain linkers and conjugates, employs modified phosphoramidites in which the modification is pre-existing. An example of such a modification would be the carbohydrate 2'-modified species (2'-F, 2'-NH<sub>2</sub>, 2'-O-alkyl, *etc.*) wherein the 2' orthoester is replaced with the desired modification 3' or 5' terminal modifications could also be introduced such as fluoroscein derivatives, Dabsyl, cholesterol, cyanine derivatives or polyethylene glycol. Certain internucleotide bond modifications would also be introduced via the incoming reactive nucleoside intermediate. Examples of the resultant internucleotide bond modification include but are not limited to methylphosphonates, phosphoramidates, phosphorothioates or phosphorodithioates.

Many modifiers can be employed using the same or similar cycles. Examples in this class would include, for example, 2-aminopurine, 5-methyl cytidine, aminoallyl uridine, diaminopurine, 2-O-alkyl, multi-atom spacers, single monomer spacers, 2'-aminonucleosides, 2'-fluoro nucleosides, 5-iodouridine, 4-thiouridine, acridines, 5-bromouridine, 5-fluorocytidine, 5-fluorouridine, 5-iodouridine, 5-iodocytidine, 5-biotin-thymidine, 5-fluoroscein -thymidine, inosine, pseudouridine, abasic monomer, nebularane, deazanucleoside, pyrene nucleoside, azanucleoside, *etc.* Often the rest of the steps in the synthesis would remain the same with the exception of modifications that introduce substituents that are labile to standard deprotection conditions. Here modified conditions would be employed that do not affect the substituent. Second, certain internucleotide bond modifications require an alteration of the oxidation step to allow for their introduction. Examples in this class include

phosphorothioates and phosphorodithioates wherein oxidation with elemental sulfur or another suitable sulfur transfer agent is required. Third, certain conjugates and modifications are introduced by "post-synthesis" process, wherein the desired molecule is added to the biopolymer after solid phase synthesis is complete. An  
5 example of this would be the addition of polyethylene glycol to a pre-synthesized oligonucleotide that contains a primary amine attached to a hydrocarbon linker. Attachment in this case can be achieved by using a N-hydroxy- succinimidyl ester of polyethylene glycol in a solution phase reaction.

10 While this outlines the most preferred method for synthesis of synthetic RNA and its analogs, any nucleic acid synthesis method that is capable of assembling these molecules could be employed in their assembly. Examples of alternative methods include 5'-DMT-2'-TBDMS and 5'-DMT-2'-TOM synthesis approaches. Some 2'-O-methyl, 2'-F and backbone modifications can be introduced in transcription  
15 reactions using modified and wild type T7 and SP6 polymerases, for example.

### SYNTHESIZING MODIFIED RNA

The following guidelines are provided for synthesis of modified RNAs, and  
20 can readily be adapted to use on any of the automated synthesizers known in the art.

#### 3' TERMINAL MODIFICATIONS

There are several methods for incorporating 3' modifications. The 3' modification can be anchored or "loaded" onto a solid support of choice using  
25 methods known in the art. Alternatively, the 3' modification may be available as a phosphoramidite. The phosphoramidite is coupled to a universal support using standard synthesis methods where the universal support provides a hydroxyl at which the 3' terminal modification is created by introduction of the activated phosphoramidite of the desired terminal modification. According to another method,  
30 the 3' modification could be introduced post synthetically after the polynucleotide is removed from the solid support. The free polynucleotide initially has a 3' terminal hydroxyl, amino, thiol, or halogen that reacts with an appropriately activated form of the modification of choice. Examples include but are not limited to N-hydroxy succinimidyl ester, thioether, disulfide, malimido, or haloalkyl reactions. This

modification now becomes the 3' terminus of the polynucleotide. Examples of modifications that can be conjugated post synthetically can be but are not limited to fluorosceins, acridines, TAMRA, dabsyl, cholesterol, polyethylene glycols, multi-atom spacers, cyanines, lipids, carbohydrates, fatty acids, steroids, peptides, or  
5 polypeptides.

### 5' TERMINAL MODIFICATIONS

There are a number of ways to introduce a 5' modification into a polynucleotide. For example, a nucleoside having the 5' modification can be  
10 purchased and subsequently activated to a phosphoramidite, or the phosphoramidite having the 5' modification may be commercially available. Then, the activated nucleoside having the 5' modification is employed in the cycle just as any other activated nucleoside may be used. However, not all 5' modifications are available as phosphoramidites. In such an event, the 5' modification can be introduced in an  
15 analogous way to that described for 3' modifications above.

### THIOATES

Polynucleotides having one or more thioate moieties, such as phosphorothioate linkages, were made in accordance with the synthesis cycle  
20 described above and illustrated in **Figure 3**. However, in place of the t-butyl hydroperoxide oxidation step, elemental sulfur or another sulfurizing agent was used.

### 5'-THIO MODIFICATIONS

Monomers having 5' thiols can be purchased as phosphoramidites from  
25 commercial suppliers such as Glen Research. These 5' thiol modified monomers generally bear trityl protecting groups. Following synthesis, the trityl group can be removed by any method known in the art.

### OTHER MODIFICATIONS

30 For certain modifications, the steps of the synthesis cycle will vary somewhat. For example, where the 3' end has an inverse dT (wherein the first base is attached to the solid support through the 5'-hydroxyl and the first coupling is a 3'-3' linkage) detritylation and coupling occurs more slowly, so extra detritylating reagent, such as dichloroacetic acid (DCA), should be used and coupling time should be increased to

300 seconds. Some 5' modifications may require extended coupling time. Examples include cholesterol, fluorophores such as Cy3 or Cy5 biotin, dabsyl, amino linkers, thio linkers, spacers, polyethylene glycol, phosphorylating reagent, BODIPY, or photocleavable linkers.

5

It should be noted that if a polynucleotide is to have only a single modification, that modification can be most efficiently carried out manually by removing the support having the partially built polynucleotide on it, manually coupling the monomer having the modification, and then replacing the support in the automated synthesizer and resuming automated synthesis.

10

## **EXAMPLE 2**

### **DEPROTECTION AND CLEAVAGE OF SYNTHESIZED OLIGOS FROM THE SUPPORT**

15

Cleaving can be done manually or in an automated process on a machine. Cleaving of the protecting moiety from the internucleotide linkage, for example a methyl group, can be achieved by using any suitable cleaving agent known in the art, for example, dithiolate or thiophenol. One molar dithiolate in DMF is added to the solid support at room temperature for 10 to 20 minutes. The support is then thoroughly washed with, for example, DMF, then water, then acetonitrile. Alternatively a water wash followed by a thorough acetonitrile will suffice to remove any residual dithioate.

20

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Cleavage of the polynucleotide from the support and removal of exocyclic base protection can be done with 40% aqueous N-methylamine (NMA), followed by heating to 55 degrees Centigrade for twenty minutes. Once the polynucleotide is in solution, the NMA is carefully removed from the solid support. The solution containing the polynucleotide is then dried down to remove the NMA under vacuum. Further processing, including duplexing, desalting, gel purifying, quality control, and the like can be carried out by any method known in the art.

30

For some modifications, the NMA step may vary. For example, for a 3' amino modification, the treatment with NMA should be for forty minutes at 55 degrees Centigrade. Puromycin, 5' terminal amino linker modifications, and 2' amino

35

nucleoside modifications are heated for 1 hour after addition of 40% NMA. Oligonucleotides modified with Cy5 are treated with ammonium hydroxide for 24 hours while protected from light.

## 5 PREPARATION OF CLEAVE REAGENTS

HPLC grade water and synthesis grade acetonitrile are used. The dithiolate is pre-prepared as crystals. Add 4.5 grams of dithiolate crystals to 90 mL of DMF. Forty percent NMA can be purchased, ready to use, from a supplier such as Sigma Aldrich Corporation.

10

## ANNEALING SINGLE STRANDED POLYNUCLEOTIDES

Single stranded polynucleotides can be annealed by any method known in the art, employing any suitable buffer. For example, equal amounts of each strand can be mixed in a suitable buffer, such as, for example, 50 mM HEPES pH 7.5, 100 mM  
15 potassium chloride, 1 mM magnesium chloride. The mixture is heated for one minute at 90 degrees Centigrade, and allowed to cool to room temperature. In another example, each polynucleotide is separately prepared such that each is at 50 micromolar concentration. Thirty microliters of each polynucleotide solution is then added to a tube with 15 microliters of 5X annealing buffer, wherein the annealing  
20 buffer final concentration is 100 mM potassium chloride, 30 mM HEPES-KOH pH 7.4 and 2 mM magnesium chloride. Final volume is 75 microliters. The solution is then incubated for one minute at 90 degrees Centigrade, spun in a centrifuge for 15 seconds, and allowed to incubate at 37 degrees Centigrade for one hour, then allowed to come to room temperature. This solution can then be stored frozen at minus 20  
25 degrees Centigrade and freeze thawed up to five times. The final concentration of the duplex is 20 micromolar. An example of a buffer suitable for storage of the polynucleotides is 20 mM KCl, 6 mM HEPES pH 7.5, 0.2 mM MgCl<sub>2</sub>. All buffers used should be RNase free.

## 30 REMOVAL OF THE ORTHOESTER MOIETY

If desired, the orthoester moiety or moieties may be removed from the polynucleotide by any suitable method known in the art. One such method employs a volatile acetic acid-tetramethylenediamine (TEMED) pH 3.8 buffer system that can be removed by lyophilization following removal of the orthoester moiety or moieties.

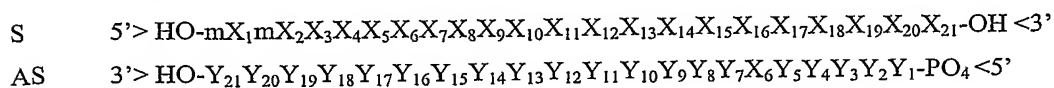
Deprotection at a pH higher than 3.0 helps minimize the potential for acid-catalyzed cleavage of the phosphodiester backbone. For example, deprotection can be achieved using 100 mM acetic acid adjusted to pH 3.8 with TEMED by suspending the orthoester protected polynucleotide and incubating it for 30 minutes at 60 degrees Centigrade. The solution is then lyophilized or subjected to a SpeedVac to dryness prior to use. If necessary, desalting following deprotection can be performed by any method known in the art, for example, ethanol precipitation or desalting on a reversed phase cartridge.

### EXAMPLE 3 siRNAs SYNTHESIZED FOR USE IN RNA INTERFERENCE

Nineteen-mer siRNAs having a di-dT overhang were synthesized using Dharmacon, Inc.'s proprietary ACE chemistry, and were designed and used in accordance with the invention described herein. "SEAP" refers to human secreted alkaline phosphatase; "human cyclo" refers to human cyclophilin B; an asterisk between nucleotide units refers to a modified internucleotide linkage that is a phosphorothioate linkage; the structure 2'-F-C or 2'-F-U refers to a nucleotide unit having a fluorine atom attached to the 2' carbon of a ribosyl moiety; the structure 2'-N-C or 2'-N-U refers to a nucleotide unit having an -NH<sub>2</sub> group attached to the 2' carbon of a ribosyl moiety; the structure 2'-OME-C or 2'-OME-U refers to a nucleotide unit having a 2'-O-methyl modification at the 2' carbon of a ribosyl moiety of either Cs or Us, respectively; dG, dU, dA, dC, and dT refer to a nucleotide unit that is deoxy with respect to the 2' position, and instead has a hydrogen attached to the 2' carbon of the ribosyl moiety. Unless otherwise indicated, all nucleotide units in the list below are ribosyl with an -OH at the 2' carbon.

### SYNTHESIS OF siRNA DUPLEX OF GENERAL FORMULA I

General Formula I:



where X<sub>q</sub> and Y<sub>q</sub> are ribonucleosides including rA, rC, rG, or rU;

$mX_q$  are 2'-O-methyl nucleosides including 2'-O-methyl-rA, 2'-O-methyl-rC, 2'-O-methyl-rG, and 2'-O-methyl-rU;

S is the sense strand of the siRNA duplex;

and AS is the antisense strand of the siRNA duplex.

5

Each strand (S and AS) of the duplex is separately chemically synthesized using the procedures described in U.S. Patent 6,008,400; U.S. Patent 6,111,086; U.S. Patent 6,590,093; Scaringe (2000) *Methods in Enzymology* 317:3-18; Scaringe (2001) *Methods* 23(3):206-217. Briefly, the procedures utilize a solid polystyrene support to which the 3'-most nucleoside ( $X_{21}$  or  $Y_{21}$ ) has been covalently tethered. Nucleosides are then added sequentially in a sequence-specific manner (3' to 5') to the support-bound species using repetitive cycles. Thus, the first cycle adds  $X_{20}$  to  $X_{21}$  or  $Y_{20}$  to  $Y_{21}$ ; the second cycle adds  $X_{19}$  to  $X_{20}X_{21}$  or  $Y_{19}$  to  $Y_{20}Y_{21}$ ; and so on. Each cycle consists of four steps: deprotection of the 5'-hydroxyl group of the support-bound species; coupling of a reactive derivative of the incoming nucleoside to the 5'-hydroxyl group of support-bound species; capping of unreacted 5'-hydroxyl groups; and oxidation of the internucleotide linkage. For  $X_q$  ( $q = 3$  to 20) or  $Y_q$  ( $q = 1$  to 20) = a ribonucleoside, the reactive derivative is a 5'-silyl-2'-orthoester-3'-phosphoramidite, in particular, a 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)silyl-2'-O-*bis*(2-acetoxyethyl)orthoformyl-3'-O-(N,N-diisopropyl)methyl phosphoramidite (Figure 15).

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20

Duplexes of General Formula I have 2'-O-methyl nucleosides in positions 1 and 2 ( $mX_q$ ,  $q = 1$  and 2) of the sense strand. These modified nucleosides are incorporated into S using the sequence-appropriate 5'-silyl-2'-O-methyl-3'-phosphoramidites, in particular, 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)-silyl-2'-O-methyl-3'-O-(N,N-diisopropyl)methyl phosphoramidites (Figure 16), and the same reaction cycle utilized for ribonucleoside incorporation described above.

25

Duplexes of General Formula I have a phosphate moiety on the 5'-terminus of the antisense strand. This phosphate group is introduced chemically using N,N-diisopropylamino-*bis*(2-cyanoethyl) phosphoramidite (Figure 17) and the same reaction cycle utilized for ribonucleoside incorporation described above.

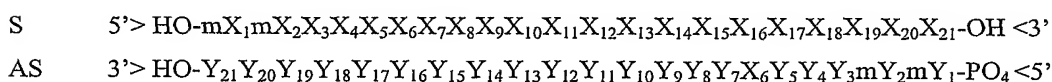
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Following chain assembly, the fully protected oligonucleotide is treated with disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate to remove the methyl groups from the internucleotide phosphate linkages. The oligonucleotide is then cleaved from the support and the base protecting groups and the 2-cyanoethyl groups on the 5'-phosphate are removed by treatment with aqueous N-methylamine, first at room temperature and then at 55°C, followed by drying under vacuum. At this point, the crude oligonucleotide is analyzed for quality by ion exchange HPLC and/or MALDI-TOF mass spectrometry, and gel purified if necessary. The oligonucleotide is then dissolved in RNase-free water or buffer and quantified using ultraviolet spectroscopy.

In order to form the duplex siRNA from the component complementary strands, equal quantities of the sense strand and antisense strand are mixed. Since the oligonucleotides retain the 2'-orthoester protection at this point, they are treated with mild acid at 55°C, which treatment removes these protecting groups. The deprotected strands are annealed to form the duplex by allowing the deprotection solution to cool slowly to room temperature. Finally, the duplex is desalted by precipitating it with ethanol, and the purified duplex is dissolved in RNase-free water and quantified by ultraviolet spectroscopy. The quality of the duplexing process is assessed by native gel electrophoresis.

## SYNTHESIS OF siRNA DUPLEX OF GENERAL FORMULA II

General Formula II:



where  $X_q$  and  $Y_q$  are ribonucleosides including rA, rC, rG, or rU;

$mX_q$  and  $mY_q$  are 2'-O-methyl nucleosides including 2'-O-methyl-rA, 2'-O-methyl-rC, 2'-O-methyl-rG, and 2'-O-methyl-rU;

S is the sense strand of the siRNA duplex;

and AS is the antisense strand of the siRNA duplex.

Each strand (S and AS) of the duplex is separately chemically synthesized using the procedures described in U.S. Patent 6,008,400; U.S. Patent No. 6,111,086;



U.S. Patent No. 6,590,093; Scaringe (2000) *Methods in Enzymology* 317:3-18; Scaringe (2001) *Methods* 23(3):206-217. Briefly, the procedures utilize a solid polystyrene support to which the 3'-most nucleoside ( $X_{21}$  or  $Y_{21}$ ) has been covalently tethered. Nucleosides are then added sequentially in a sequence-specific manner (3' to 5') to the support-bound species using repetitive cycles. Thus, the first cycle adds  $X_{20}$  to  $X_{21}$  or  $Y_{20}$  to  $Y_{21}$ ; the second cycle adds  $X_{19}$  to  $X_{20}X_{21}$  or  $Y_{19}$  to  $Y_{20}Y_{21}$ ; and so on. Each cycle consists of four steps: deprotection of the 5'-hydroxyl group of the support-bound species; coupling of a reactive derivative of the incoming nucleoside to the 5'-hydroxyl group of support-bound species; capping of unreacted 5'-hydroxyl groups; and oxidation of the internucleotide linkage. For  $X_q$  ( $q = 3$  to 20) or  $Y_q$  ( $q = 3$  to 20) = a ribonucleoside, the reactive derivative is a 5'-silyl-2'-orthoester-3'-phosphoramidite, in particular, a 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)silyl-2'-O-*bis*(2-acetoxyethyl)orthoformyl-3'-O-(N,N-diisopropyl)methyl phosphoramidite (**Figure 15**).

Duplexes of General Formula II have 2'-O-methyl nucleosides in positions 1 and 2 ( $mX_q$ ,  $q = 1$  and 2) of the sense strand and in positions 1 and 2 ( $mY_q$ ,  $q = 1$  and 2) of the antisense strand. These modified nucleosides are incorporated into S and AS using the sequence-appropriate 5'-silyl-2'-O-methyl-3'-phosphoramidites, in particular, 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)-silyl-2'-O-methyl-3'-O-(N,N-diisopropyl)methyl phosphoramidites (**Figure 16**), and the same reaction cycle utilized for ribonucleoside incorporation described above.

Duplexes of General Formula II have a phosphate moiety on the 5'-terminus of the antisense strand. This phosphate group is introduced chemically using N,N-diisopropylamino-*bis*(2-cyanoethyl) phosphoramidite (**Figure 17**) and the same reaction cycle utilized for ribonucleoside incorporation described above.

Following chain assembly, the fully protected oligonucleotide is treated with disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate to remove the methyl groups from the internucleotide phosphate linkages. The oligonucleotide is then cleaved from the support and the base protecting groups and the 2-cyanoethyl groups on the 5'-phosphate are removed by treatment with aqueous N-methylamine, first at room temperature and then at 55°C, followed by drying under vacuum. At this

point, the crude oligonucleotide is analyzed for quality by ion exchange HPLC and/or MALDI-TOF mass spectrometry, and gel purified if necessary. The oligonucleotide is then dissolved in RNase-free water or buffer and quantified using ultraviolet spectroscopy.

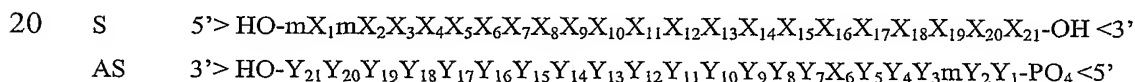
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In order to form the duplex siRNA from the component complementary strands, equal quantities of the sense strand and antisense strand are mixed. Since the oligonucleotides retain the 2'-orthoester protection at this point, they are treated with mild acid at 55°C, which treatment removes these protecting groups. The deprotected strands are annealed to form the duplex by allowing the deprotection solution to cool slowly to room temperature. Finally, the duplex is desalted by precipitating it with ethanol, and the purified duplex is dissolved in RNase-free water and quantified by ultraviolet spectroscopy. The quality of the duplexing process is assessed by native gel electrophoresis.

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### 15 SYNTHESIS OF siRNA DUPLEX OF GENERAL FORMULA III

GENERAL FORMULA III:



where X<sub>q</sub> and Y<sub>q</sub> are ribonucleosides including rA, rC, rG or rU;

mX<sub>q</sub> and mY<sub>q</sub> are 2'-O-methyl nucleosides including 2'-O-methyl-rA, 2'-O-methyl-rC, 2'-O-methyl-rG, and 2'-O-methyl-rU;

25

S is the sense strand of the siRNA duplex;

and AS is the antisense strand of the siRNA duplex.

Each strand (S and AS) of the duplex is separately chemically synthesized using the procedures described in US Patent No. 6,008,400; US Patent No. 6,111,086; US Patent No. 6,590,093; Scaringe (2000) *Methods in Enzymology* 317:3-18; Scaringe (2001) *Methods* 23(3):206-217. Briefly, the procedures utilize a solid polystyrene support to which the 3'-most nucleoside (X<sub>21</sub> or Y<sub>21</sub>) has been covalently tethered. Nucleosides are then added sequentially in a sequence-specific manner (3' to 5') to the support-bound species using repetitive cycles. Thus, the first cycle adds X<sub>20</sub> to X<sub>21</sub> or Y<sub>20</sub> to Y<sub>21</sub>; the second cycle adds X<sub>19</sub> to X<sub>20</sub>X<sub>21</sub> or Y<sub>19</sub> to Y<sub>20</sub>Y<sub>21</sub>; and so

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on. Each cycle consists of four steps: deprotection of the 5'-hydroxyl group of the support-bound species; coupling of a reactive derivative of the incoming nucleoside to the 5'-hydroxyl group of support-bound species; capping of unreacted 5'-hydroxyl groups; and oxidation of the internucleotide linkage. For  $X_q$  ( $q = 3$  to 20) or  $Y_q$  ( $q = 1$  or 3 to 20) = a ribonucleoside, the reactive derivative is a 5'-silyl-2'-orthoester-3'-phosphoramidite, in particular, a 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)silyl-2'-O-*bis*(2-acetoxyethyl)orthoformyl-3'-O-(N,N-diisopropyl)methyl phosphoramidite (Figure 15).

10 Duplexes of General Formula III have 2'-O-methyl nucleosides in positions 1 and 2 ( $mX_q$ ,  $q = 1$  and 2) of the sense strand and in position 2 ( $mY_q$ ,  $q = 2$ ) of the antisense strand. These modified nucleosides are incorporated into S and AS using the sequence-appropriate 5'-silyl-2'-O-methyl-3'-phosphoramidites, in particular, 5'-O-benzhydroxy-*bis*(trimethylsilyloxy)-silyl-2'-O-methyl-3'-O-(N,N-diisopropyl)methyl phosphoramidites (Figure 16), and the same reaction cycle  
15 utilized for ribonucleoside incorporation described above.

Duplexes of General Formula III have a phosphate moiety on the 5'-terminus of the antisense strand. This phosphate group is introduced chemically using N,N-diisopropylamino-*bis*(2-cyanoethyl) phosphoramidite (Figure 17) and the same  
20 reaction cycle utilized for ribonucleoside incorporation described above.

Following chain assembly, the fully protected oligonucleotide is treated with disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate to remove the  
25 methyl groups from the internucleotide phosphate linkages. The oligonucleotide is then cleaved from the support and the base protecting groups and the 2-cyanoethyl groups on the 5'-phosphate are removed by treatment with aqueous N-methylamine, first at room temperature and then at 55°C, followed by drying under vacuum. At this point, the crude oligonucleotide is analyzed for quality by ion exchange HPLC and/or  
30 MALDI-TOF mass spectrometry, and gel purified if necessary. The oligonucleotide is then dissolved in RNase-free water or buffer and quantified using ultraviolet spectroscopy.

In order to form the duplex siRNA from the component complementary  
35 strands, equal quantities of the sense strand and antisense strand are mixed. Since the

oligonucleotides retain the 2'-orthoester protection at this point, they are treated with mild acid at 55°C, which treatment removes these protecting groups. The deprotected strands are annealed to form the duplex by allowing the deprotection solution to cool slowly to room temperature. Finally, the duplex is desalted by precipitating it with ethanol, and the purified duplex is dissolved in RNase-free water and quantified by ultraviolet spectroscopy. The quality of the duplexing process is assessed by native gel electrophoresis.

#### TRANSFECTION

siRNA duplexes were annealed using standard buffer (50 millimolar HEPES pH 7.5, 100 millimolar KCl, 1 mM MgCl<sub>2</sub>). The transfections are done according to the standard protocol described below.

#### STANDARD TRANSFECTION PROTOCOL FOR 96 WELL AND 6 WELL PLATES: siRNAs

1. Protocols for 293 and Calu6, HeLa, MDA 75 are identical.
2. Cells are plated to be 95% confluent on the day of transfection.
3. SuperRNasin (Ambion) is added to transfection mixture for protection against RNases.
4. All solutions and handling have to be carried out in RNase free conditions.

Plate 1 0.5 –1 ml in 25 ml of media in a small flask or 1 ml in 50 ml in a big flask.

#### 96 WELL PLATE

1. Add 3 ml of 0.05 % trypsin-EDTA in a medium flask (6 in a large flask) incubate 5 min at 37 degrees C.
2. Add 7 ml (14 ml big) of regular media and pipet 10 times back and forth to re-suspend cells.
3. Take 25 microliters of the cell suspension from step 2 and 75 microliters of trypan blue stain (1:4) and place 10 microliters in a cell counter.
4. Count number of cells in a standard hemocytometer.
5. Average number of cells x 4 x 10000 is number of cells per ml.
6. Dilute with regular media to have 350 000 /ml.
7. Plate 100 microliters (35,000 cell for HEK293) in a 96 well plate.

**TRANSFECTION FOR 2 X 96 WELL PLATES (60 WELL FORMAT)**

1. OPTI-MEM 2 ml + 80 microliters Lipofectamine 2000 (1:25) + 15 microliters of SuperRNAsin (AMBION).
2. Transfer siRNA aliquots (0.8 microliters of 100 micromolar to screen (total dilution factor is 1:750, 0.8 microliters of 100 micromolar solution will give 100 nanomolar final) to the deepdish in a desired order (usually 3 columns x 6 for 60 well format or four columns by 8 for 96 well).
3. Transfer 100 microliters of OPTI-MEM.
4. Transfer 100 microliters of OPTI-MEM with Lipofectamine 2000 and SuperRNAsin to each well.
5. Leave for 20-30 min RT.
6. Add 0.55 ml of regular media to each well. Cover plate with film and mix.
7. Array out 100 x 3 x 2 directly to the cells (sufficient for two plates).

**TRANSFECTION FOR 2 X 6 WELL PLATES**

8. 8 ml OPTI-MEM + 160 microliters Lipofectamine 2000 (1:25). 30 microliters of SuperRNAsin (AMBION).
9. Transfer siRNA aliquots (total dilution factor is 1:750, 5 microliters of 100 micromolar solution will give 100 nanomolar final) to polystyrene tubes.
10. Transfer 1,300 microliters of OPTI-MEM with Lipofectamine 2000 and SuperRNAsin (AMBION).
11. Leave for 20-30 min RT.
12. Add 0.55 ml of regular media to each well. Cover plate with film and mix.
13. Transfer 2 ml to each well (sufficient for two wells).

The mRNA or protein levels are measured 24, 48, 72, and 96 hours post transfection with standard kits or Custom B-DNA sets and Quantigene kits (Bayer).

**EXAMPLE 5  
MEASUREMENT OF ACTIVITY/DETECTION**

The level of siRNA-induced RNA interference, or gene silencing, was estimated by assaying the reduction in target mRNA levels or reduction in the corresponding protein levels. Assays of mRNA levels were carried out using B-

DNA™ technology (Quantagene Corp.). Protein levels for fLUC and rLUC were assayed by STEADY GLO™ kits (Promega Corp.). Human alkaline phosphatase levels were assayed by Great EscAPe SEAP Fluorescence Detection Kits (#K2043-1), BD Biosciences, Clontech.

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For microarray analysis: HeLa cells were transfected in 6-well plates using Oligofectamine (Invitrogen) and the indicated doses of siRNA duplex. Where not specified, the concentration of siRNA was 100nM. RNA was isolated 24 hours following transfection. RNA from siRNA-transfected cells was hybridized against RNA from mock-transfected cells (treated with transfection reagent in the absence of RNA duplex). Total RNA was purified by Qiagen RNeasy kit, and processed for hybridization to microarrays containing oligonucleotides corresponding to approximately 21,000 human genes. Ratio hybridizations were performed with fluorescent label reversal to eliminate dye bias. Microarrays were either purchased from Agilent Technologies or synthesized as described in Hughes, T.R., *et al.* (2001) Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nat. Biotech.* 19: 342-347. Each row represents the expression pattern resulting from transfection of an individual siRNA. Microarray data is presented in **Figures 7-11**. Data shown are signature genes that display a difference in expression level (p value<0.01 and log<sub>10</sub> intensity >-1.5) relative to mock-transfected cells. No cuts were placed on fold change in expression. Green indicates decreased expression; red indicates increased expression. Data were analyzed using Rosetta Resolver™ software. The histogram at the top of cluster diagrams reflects the similarity of gene expression changes between different genes analyzed in the experiment.

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#### EXAMPLE 6 IDENTIFICATION OF CHEMICAL MODIFICATIONS THAT MODIFY SILENCING ACTIVITY

Using 2'-O- ACE chemistry as a platform for RNA synthesis, a modification walk consisting of one, two, or three consecutively modified nucleotides in sense (S) and antisense (AS) strands was performed on SEAP-2217, an siRNA directed against human secreted alkaline phosphatase (SEAP, SEAP-2217-sense strand 5'-GUGAUGUAUGUCAGAGAGUdTdT-3' (SEQ. ID NO. 22). Subsequently, the silencing efficiency of these modified siRNAs was evaluated by cotransfecting each

35

duplex with a SEAP expression vector (Clontech) into HEK293 cells (100nM siRNA, 50 ng/well SEAP expression vector, Lipofectamine 2000) and assaying for a decrease in target protein activity twenty-four hours after transfection. **Figure 5** shows the relationship between modification and function for 2'-O-methylated SEAP-2217 siRNA. Unmodified duplexes targeting SEAP induce >90% silencing of the SEAP gene. Single base modifications of both S and AS strands induced little or no effect on siRNA activity, suggesting that no single 2'-hydroxyl group on either strand plays an indispensable role in target specific RNAi. In contrast, a walk of dual, side-by-side, modifications identified several key positions where the introduction of modified bases interfered significantly with silencing activity. The most profound interference with function was observed when two consecutive bases (positions 1 and 2) or three consecutive bases (positions 1, 2, and 3) of the 5' end of the AS strand were modified, thus hinting of a cooperative effect between adjacent modified groups. As similar modifications of the S strand failed to alter duplex functionality, paired 2'-O-methyl modified bases enable a distinction of S and AS strands and identification of key positions for target knockdown. Moreover, these experiments identify positions within the duplex that play a key role in target (and possibly off-target) silencing.

#### EXAMPLE 7

##### ADDITIONAL ANALYSIS OF THE EFFECTS OF VARIOUS COMBINATIONS OF CHEMICAL MODIFICATIONS ON siRNA-INDUCED GENE SILENCING

To test the effects of 2'-O-methyl modifications on duplex functionality in various combinations, a series of siRNA directed against the luciferase gene (luc 8, 18, 56, 58, 63, and 81) were synthesized using 2'-O-ACE chemistry and modified to contain O-methyl groups at the 2' position of the ribose ring.

Luc 8 5'-GAAAAAUCAGAGAGAUCCU-3' (SEQ. ID NO. 23)

Luc 18 5'-UACCGGAAAACUCGACGCA-3' (SEQ. ID NO. 24)

Luc 56 5'-ACGUCGCCAGUCAAGUAAC-3' (SEQ. ID NO. 25)

Luc 58 5'-GAUUACGUCGCCAGUCAAG-3' (SEQ. ID NO. 26)

Luc 63 5'-AGAGAUCGUGGAUUACGUC-3' (SEQ. ID NO. 27)

Luc 81 5'-UGUUGUUUUGGAGCACGGA-3' (SEQ. ID NO. 28)

(Sequences listed above are the sense strand.)

Specifically, siRNA containing 2'-O-methyl modifications on the two 5'-most  
5 nucleotides of (1) the sense strand, (2) the antisense strand, or (3) both strands, were  
co-transfected along with a Luc-expression plasmid (pCMVLuc, 50ng/well) into  
HEK293 cells. Subsequently, a side-by-side comparison of the silencing ability of  
each duplex was performed to determine the effects of this modification on target  
transcript degradation.

10 Results of these studies showed that addition of the 2'-O-methyl groups only  
to the AS strand dramatically diminished the ability of the duplex to silence the target  
mRNA (see **Figure 6**). In contrast, duplexes carrying this modification on the sense  
strand performed as well (luc 58, 63, 81) or better (luc 56, 8, 18) than equivalent,  
15 unmodified siRNA, suggesting that modification of the sense strand biased strand  
selection by RISC and (in some cases) increased the effective antisense strand  
concentration. Enhanced silencing could be the result of a decrease in the binding  
affinity of RISC to the 5' sense end of the molecule (and therefore an increase in the  
availability of free RISC for association to the opposing end), decreased ability of  
20 native kinases to phosphorylate the sense strand (thus decreasing competition between  
the sense and antisense strand for access to RISC), or a decline in the ability of RISC  
to unravel the duplex from the 5'-sense end. siRNA containing 2'-O-methyl  
modifications on both strands exhibited decreased silencing abilities that were  
between the values observed for molecules that contained modifications on either  
25 single strand. One interpretation of these results is that 2'-O-methyl modifications  
lowers the binding affinity that RISC has for the modified strand. In cases where both  
strands are modified, neither strand receives an advantage over its complement, and a  
new equilibrium representing an average of the functionality of both modified  
molecules is established.

30 To test whether the diminished level of silencing observed in cells containing  
2'-O-methylated S/AS siRNA was the result of a debilitated capacity of cellular  
kinases to phosphorylate the duplexes, siRNAs carrying the 2'-O-methyl  
modifications were modified to carry a phosphate group on the 5' end of the AS



strand. Specifically, Luc siRNAs carrying 2'-O-methyl groups on either: (1) positions 1 and 2 of the 5' end of the antisense strand; or (2) positions 1 and 2 of the 5' end of both antisense and sense strands, were 5'-phosphorylated on the AS strand during synthesis. These duplexes were then introduced into HEK293 cells using previously described procedures and tested for the ability to silence the desired target. Results showed that in 83% of the cases tested (10/12), 5' phosphorylation of the antisense strand improved the silencing efficiency of the duplex over the equivalent unphosphorylated molecule (**Figure 6**). In the remaining two cases, silencing remained unchanged or was improved only marginally. These results demonstrate that the combination of 5' phosphorylation of the antisense strand and 2'-O-methylation of positions 1 and 2 of the sense and antisense strands are compatible with maintaining duplex functionality. Moreover, as dual 2'-O-methyl modifications of positions 1 and 2 of a strand (in the absence of 5' phosphorylation of the terminal position) severely compromises the silencing ability of the unphosphorylated strand, this modification pattern (2'-O-methyl modification of positions 1 and 2 of the sense strand, 2'-O-methyl modification of positions 1 and 2 of the antisense strand, plus 5' phosphorylation of the antisense strand) identifies a strategy for eliminating sense strand off-targets without compromising on-target knockdown. Moreover, the potential effect of 2'-O-methylation on other steps led the authors to consider that the possibility that said modifications might also alter the ability of RISC to distinguish between intended targets that have 100% homology with the antisense strand and off-targets that have lesser amounts of homology.

#### EXAMPLE 8

##### IDENTIFICATION OF CHEMICAL MODIFICATION PATTERNS THAT ELIMINATE, MINIMIZE, OR ALTER OFF-TARGET EFFECTS GENERATED BY siRNA

To determine whether siRNA containing (2'-O-methyl modification of positions 1 and 2 of the sense strand, 2'-O-methyl modification of positions 1 and 2 of the antisense strand, plus 5' phosphorylation of the antisense strand) modification pattern had the same or altered off-target effects, siRNA targeting IGFR1 (IGFR1-73) were transfected into cells in unmodified and modified states. As shown in **Figure 7**, while the unmodified version of the siRNA induced significant off-target gene modulation, the modified form down regulated a much more limited subset. These findings were consistently observed across a broad range of siRNA tested (See **Figure**

8 for MAPK14-153 heat map and MPHOSH1-202, and **Figure 9** for summation of results on 8 different siRNA targeting 4 genes). In all of these cases, silencing by the fully modified molecule was roughly equivalent to the unmodified molecule.

- 5 To determine whether the number or position of 2'-O-methyl modifications was important for the observed increased specificity, the inventors performed a walk of chemical modifications across the MAPK14-153. All of the duplexes in these studies with the exception of duplex D (unmodified duplex) and duplex F (2'-O-methyl modified on positions 1 and 2 of the antisense strand, no modification on the
- 10 antisense strand) contain paired 2'-O-methyl modifications on positions 1 and 2 of the sense strand. Furthermore, all duplexes in this study (D → R) contain a phosphate group on the 5' end of the AS strand. In addition, the complementary strand in the remaining configurations (E, G → Q) contain the following modifications:
- 15 E: 2'-O-methyl modification of positions 1 and 2 of the AS strand  
 G: 2'-O-methyl modification of positions 2 and 3 of the AS strand  
 H: 2'-O-methyl modification of positions 3 and 4 of the AS strand  
 I: 2'-O-methyl modification of positions 4 and 5 of the AS strand  
 J: 2'-O-methyl modification of positions 5 and 6 of the AS strand  
 20 K: 2'-O-methyl modification of positions 6 and 7 of the AS strand  
 L: 2'-O-methyl modification of positions 7 and 8 of the AS strand  
 M: 2'-O-methyl modification of positions 8 and 9 of the AS strand  
 N: 2'-O-methyl modification of positions 9 and 10 of the AS strand  
 O: 2'-O-methyl modification of positions 10 and 11 of the AS strand  
 25 P: 2'-O-methyl modification of position 1 of the AS strand  
 Q: 2'-O-methyl modification of position 2 of the AS strand

As shown in **Figure 10a**, only three modification patterns, E, G, and Q exhibit significant reductions in off-target effects. As the common element amongst all three

30 of these molecules is the modification at position 2 on the antisense strand, this position is identified as a key element for eliminating off-targets.

To confirm this finding, three additional siRNAs targeting MAPK14, KNTC2, and STK6 were designed to contain: (1) the modifications on positions 1 and 2 of the

sense strand; (2) the modifications on positions 1 and 2 of the sense strand plus the modifications on position 1 of the antisense strand; (3) the modifications on positions 1 and 2 of the sense strand plus the modifications on position 2 of the antisense strand; (4) the modifications on positions 1 and 2 of the sense strand plus the modifications on positions 1 and 2 of the antisense strand. The off-target effects generated by these molecules were compared with unmodified siRNA. In all of the cases studied, the antisense strand also contains a phosphate group on the 5' carbon of the 5'-terminal nucleotide. siRNA targeting MAPK14, KNTC2, and STK6 (MAPK14, 5' 193 CCUACAGAGAACUGCGGUU-3' (SEQ. ID NO. 29), sense sequence; KNTC2, 5' GGCUUCCUUACAAGGAGAU-3' (SEQ. ID NO. 30), sense sequence; and STK6, 5' CGGGUCUUGUGUCCUCAA-3' (SEQ. ID NO. 31), sense sequence) all show significant levels of off-target effects when they are unmodified (**Figure 10b**). In contrast, addition of the following modification pattern: 2'-O-methyl modification of sense nucleotides 1 and 2, plus 2'-O-methyl modification of antisense nucleotides 1 and 2 (or just 2), plus phosphorylation of the 5' carbon of the first antisense nucleotide, was sufficient to eliminate the majority of off-target effects. These studies demonstrate the fundamental importance of position 2 in limiting off-target effects and the ability of the chemical modification pattern described in embodiment 1 to reduce and/or eliminate these effects.

#### EXAMPLE 9

##### ASSESSMENT OF BASE-PAIR MISMATCHES TO ELIMINATE OFF-TARGET EFFECTS: A COMPARISON WITH CHEMICALLY MODIFIED siRNA

To further explore the importance of position 2 in off-target effects, base pair mismatches were incorporated into siRNA targeting the MAPK14 gene. Duplexes carrying single base pair mismatches (between the antisense strand and the target site of the target) were then compared with siRNA carrying paired chemical modifications (2'-O-methyl modification) at positions across the molecule (*i.e.*, positions 1 and 2, 2 and 3, 3 and 4, *etc.*... of the antisense strand). Results of these experiments are provided in **Figure 11** and demonstrate several important points. First, as observed previously, chemical modification of positions 1 and 2 have the greatest effect on eliminating the off-target signature, while paired 2'-O-methyl modifications at other positions provided lesser amounts of off-target silencing. Surprisingly, introduction of a basepair mismatches at various positions across the duplex provided variable results,

depending upon the position of the mismatch. A mismatch at position 1 failed to eliminate the off-target signature of the unmodified molecule and led to additional/more enhanced down regulation of some of the genes. Introduction of base-pair mismatches at positions 2-7 eliminated a substantial portion of the signature generated by unmodified duplexes, but frequently led to altered patterns of expression of other off-targeted genes. For MAPK14-153, this was particularly evident when mismatches were introduced at position 4 of the antisense strand. Together, these studies demonstrate that while both base pair mismatches and embodiment 1 chemical modification patterns can alter siRNA off-target effects, chemical modification patterns are superior due to the fact that a secondary signature does not replace the pattern observed in unmodified molecules.

#### EXAMPLE 10

##### DEMONSTRATION THAT siRNA OFF-TARGET EFFECTS GENERATE OBSERVABLE PHENOTYPES: TOXIC siRNA

The importance of the present invention became evident when it was recognized that off-target effects can induce phenotypes that were not associated with target knockdown. This phenomenon became apparent in a study of siRNA induced off-targeting and cellular toxicity. A population of randomly selected siRNA derived from a siRNA walk targeting DBI (NM\_020548, position 202-291) were assessed for the ability to induce toxicity. The collection of targeting siRNA consisted of 90 individual (19 nt) duplexes and covered the respective region in single base steps. Duplexes were transfected into HeLa cells (10,000 cells per well, 10nM siRNA) using Lipofectamine 2000 (Invitrogen) and a threshold of 75% cell viability was used as an arbitrary cutoff to distinguish toxic from nontoxic sequences. The survival of cells after treatment was determined by Alamar Blue (BioSource Int.) cytotoxicity assay according to manufacturers instructions.

siRNA transfected under these conditions were observed to induce varying levels of cellular toxicity. Overall, 14 out of 90 siRNA duplexes (15.5%) were found to decrease cellular viability below 75% (**Figure 12a**). As examples of both toxic and non-toxic siRNA could be found to induce strong DBI silencing, the relative cytotoxicity of each siRNA was unrelated to target specific knockdown.

Independent confirmation of the siRNA induced toxicity was obtained from analysis of a separate collection of 48 functional (>70% silencing) siRNA targeting 12 different genes (ARAF1, NM\_001654, MAP2K1, NM\_002755, MAP2K2 ,  
5 NM\_030662, PI3K-CA , NM\_006218, Pi3K-CB, NM\_006219 Bcl2 , NM\_000633, Bcl3 , NM\_005178, MAPK1, NM\_002745, MAPK3, NM\_002746, AR , NM\_000044, SRD5a1, NM\_001047, SRD5a2, NM\_000348, four siRNAs per gene, **Figure 12b**). Only twelve of the forty-eight sequences (25%) decreased cellular viability below 75%. An exemplary group of duplexes from this collection are shown  
10 in **Figure 12c**. While all eight duplexes targeting MAPK1 and MAPK2 show greater than 80% gene silencing, only a single siRNA in each quartet reduces cell viability below 75% (MAPK1-d4 and MAPK2-d3). Thus, as the remaining siRNAs in each group were equally functional in the ability to silence the target, but non-toxic, the toxicity induced by MAP2K1-d4 and MAP2K2-d3 is unrelated to target knockdown.  
15 Furthermore, the relative level of toxicity was found to be dependent upon the concentration of the siRNA during transfection (**Figure 12d**). As both siRNA induced toxicity and off-target effects show a dependence on siRNA concentration, it was predicted that siRNA induced toxicity was an off-target effect.

20 The linear display of the distribution of toxic siRNA along the DBI walk showed that the dispersal of these sequences was frequently non-random (*i.e.*, clustered) and suggested the presence of one or more motifs that were responsible for the observed toxicity (**Figure 12a**, boxed areas). Subsequent analysis of the toxic sequences from the random functional siRNA set revealed that all twelve sequences  
25 contained either an AAA/UUU or GCCA/UGGC motif. To test whether a correlation existed between the presence of these motifs and toxicity, three additional, randomly selected, groups of siRNA that contained either AAA/UUU motifs, GCCA/UGGC motifs, or neither motif, were chosen and tested for the ability to induce cell death. As shown in **Figures 13a and 13b**, siRNA containing the AAA/UUU and  
30 GCCA/UGGC motifs exhibited a higher probability of inducing toxicity (56 % and 53 %, respectively) than non-motif containing siRNA (**Figure 13c**, 6 %). As the T-Test p-value for these two samples was  $1.3 \times 10^{-7}$  these findings strongly support the notion that a strong correlation exists between siRNA induced cellular toxicity and delivery of duplexes containing the AAA/UUU or GCCA/UGGC motifs. The target

sequences for the siRNAs used to generate the data of **Figure 12 and 13** are provided in **Table I**. Modifications of corresponding siRNAs, when employed, are indicated in the examples and figure descriptions.

TABLE I SEQUENCES USED FOR THE DATA OF FIGURES 12-14					
FIG.	ACCESSION #	GENE NAME	DESIGNATION	SIRNA TARGET SEQUENCE	SEQ. ID NO.
12A					
	NM_020548	DBI			
			1	ACGGGCAAGGCCAAGUGGG	32
			2	CGGGCAAGGCCAAGUGGGA	33
			3	GGGCAAGGCCAAGUGGGAU	34
			4	GGCAAGGCCAAGUGGGAUG	35
			5	GCAAGGCCAAGUGGGAUGC	36
			6	CAAGGCCAAGUGGGAUGCC	37
			7	AAGGCCAAGUGGGAUGCCU	38
			8	AGGCCAAGUGGGAUGCCUG	39
			9	GGCCAAGUGGGAUGCCUGG	40
			10	GCCAAGUGGGAUGCCUGGA	41
			11	CCAAGUGGGAUGCCUGGAA	42
			12	CAAGUGGGAUGCCUGGAAU	43
			13	AAGUGGGAUGCCUGGAAUG	44
			14	AGUGGGAUGCCUGGAAUGA	45
			15	GUGGGAUGCCUGGAAUGAG	46
			16	UGGGAUGCCUGGAAUGAGC	47
			17	GGGAUGCCUGGAAUGAGCU	48
			18	GGAUGCCUGGAAUGAGCUG	49
			19	GAUGCCUGGAAUGAGCUGA	50
			20	AUGCCUGGAAUGAGCUGAA	51
			21	UGCCUGGAAUGAGCUGAAA	52
			22	GCCUGGAAUGAGCUGAAAG	53
			23	CCUGGAAUGAGCUGAAAGG	54
			24	CUGGAAUGAGCUGAAAGGG	55
			25	UGGAAUGAGCUGAAAGGGA	56
			26	GGAAUGAGCUGAAAGGGAC	57
			27	GAAUGAGCUGAAAGGGACU	58
			28	AAUGAGCUGAAAGGGACUU	59
			29	AUGAGCUGAAAGGGACUUC	60
			30	UGAGCUGAAAGGGACUUCC	61
			31	GAGCUGAAAGGGACUUCCA	62
			32	AGCUGAAAGGGACUUCCAA	63
			33	GCUGAAAGGGACUUCCAAG	64
			34	CUGAAAGGGACUUCCAAGG	65
			35	UGAAAGGGACUUCCAAGGA	66
			36	GAAAGGGACUUCCAAGGAA	67
			37	AAAGGGACUUCCAAGGAAG	68

**TABLE I**  
**SEQUENCES USED FOR THE DATA OF FIGURES 12-14**

FIG.	ACCESSION #	GENE NAME	DESIGNATION	SIRNA TARGET SEQUENCE	SEQ. ID NO.
			38	AAGGGACUUCCAAGGAAGA	69
			39	AGGGACUUCCAAGGAAGAU	70
			40	GGGACUUCCAAGGAAGAUG	71
			41	GGACUUCCAAGGAAGAUGC	72
			42	GACUUCCAAGGAAGAUGCC	73
			43	ACUUCCAAGGAAGAUGCCA	74
			44	CUUCCAAGGAAGAUGCCA	75
			45	UUCCAAGGAAGAUGCCAUG	76
			46	UCCAAGGAAGAUGCCAUGA	77
			47	CCAAGGAAGAUGCCAUGAA	78
			48	CAAGGAAGAUGCCAUGAAA	79
			49	AAGGAAGAUGCCAUGAAAG	80
			50	AGGAAGAUGCCAUGAAAGC	81
			51	GGAAGAUGCCAUGAAAGCU	82
			52	GAAGAUGCCAUGAAAGCUU	83
			53	AAGAUGCCAUGAAAGCUUA	84
			54	AGAUGCCAUGAAAGCUUAC	85
			55	GAUGCCAUGAAAGCUUACA	86
			56	AUGCCAUGAAAGCUUACAU	87
			57	UGCCAUGAAAGCUUACAUC	88
			58	GCCAUGAAAGCUUACAUCA	89
			59	CCAUGAAAGCUUACAUCAA	90
			60	CAUGAAAGCUUACAUCAAC	91
			61	AUGAAAGCUUACAUCAACA	92
			62	UGAAAGCUUACAUCAACAA	93
			63	GAAAGCUUACAUCAACAAA	94
			64	AAAGCUUACAUCAACAAAG	95
			65	AAGCUUACAUCAACAAAGU	96
			66	AGCUUACAUCAACAAAGUA	97
			67	GCUUACAUCAACAAAGUAG	98
			68	CUUACAUCAACAAAGUAGA	99
			69	UUACAUCAACAAAGUAGAA	100
			70	UACAUCAACAAAGUAGAAG	101
			71	ACAUCAACAAAGUAGAAGA	102
			72	CAUCAACAAAGUAGAAGAG	103
			73	AUCAACAAAGUAGAAGAGC	104
			74	UCAACAAAGUAGAAGAGCU	105
			75	CAACAAAGUAGAAGAGCUA	106
			76	AACAAAGUAGAAGAGCUAA	107
			77	ACAAAGUAGAAGAGCUAAA	108
			78	CAAAGUAGAAGAGCUAAAG	109
			79	AAAGUAGAAGAGCUAAAGA	110
			80	AAGUAGAAGAGCUAAAGAA	111
			81	AGUAGAAGAGCUAAAGAAA	112
			82	GUAGAAGAGCUAAAGAAAA	113

**TABLE I**  
**SEQUENCES USED FOR THE DATA OF FIGURES 12-14**

FIG.	ACCESSION #	GENE NAME	DESIGNATION	SIRNA TARGET SEQUENCE	SEQ. ID NO.
			83	UAGAAGAGCUAAAGAAAAA	114
			84	AGAAGAGCUAAAGAAAAA	115
			85	GAAGAGCUAAAGAAAAAAU	116
			86	AAGAGCUAAAGAAAAAAUA	117
			87	AGAGCUAAAGAAAAAAUAC	118
			88	GAGCUAAAGAAAAAAUACG	119
			89	AGCUAAAGAAAAAAUACGG	120
			90	GCUAAAGAAAAAAUACGGG	121
<b>12B</b>					
	NM_000633	Bcl2	Bcl2 2	GAAGUACAUCCAUUAUAAG	122
	NM_002745	MAPK1	MAPK1 2	AAACAGAUUUUACAAGCU	123
	NM_006219	PI3K Cb	PI3K Cb 4	UUUCAAGUGUCUCCUAAUA	124
	NM_001654	ARaf1	Raf1 2	GCAAAGAACAUCAUCCAUA	125
	NM_002755	MAP2K1	MAP2K1 2	GCAGAGAGAGCAGAUUUGA	126
	NM_000044	AR	AR 3	UCAAGGAACUCGAUCGUAU	127
	NM_001654	ARaf1	Raf1 3	GACAUGAAAUCCAACAAUA	128
	NM_006219	PI3K Cb	PI3K Cb 2	UCAAGUGUCUCCUAAUAUG	129
	NM_030662	MAP2K2	MAP2K2 1	CAAAGACGAUGACUUCGAA	130
	NM_030662	MAP2K2	MAP2K2 4	GGAAGCUGAUCCACCUUGA	131
	NM_002745	MAPK1	MAPK1 3	CAAGAGGAUUGAAGUAGAA	132
	NM_002745	MAPK1	MAPK1 1	CCAAAGCUCUGGACUUAUU	133
	NM_000633	Bcl2	Bcl2 3	GUACGACAACCGGGAGAU	134
	NM_000633	Bcl2	Bcl2 4	AGAUAGUGAUGAAGUACAU	135
	NM_000633	Bcl2	Bcl2 1	GGGAGAUAGUGAUGAAGUA	136
	NM_000044	AR	AR 4	GAAUGAUUGCACUAUUGA	137
	NM_001654	ARaf1	Raf1 1	GCACGGAGAUGUUGCAGUA	138
	NM_000348	SRD5A2	SRD5A2 1	GCUACUAUCUGAUUUACUG	139
	NM_000044	AR	AR 2	CAAGGGAGGUACACCAA	140
	NM_002755	MAP2K1	MAP2K1 3	GAGGUUCUCUGGAUCAAGU	141
	NM_002746	MAPK3	MAPK3 4	GCUACACGCAGUUGCAGUA	142
	NM_002746	MAPK3	MAPK3 2	AGACUGACCUGUACAAGUU	143
	NM_030662	MAP2K2	MAP2K2 2	GAUCAGCAUUUGCAUGGAA	144
	NM_000044	AR	AR-1	GGAACUCGAUCGUUAUCAU	145
	NM_006219	PI3K Cb	PI3K Cb-1	CGACAAGACUGCCGAGAGA	146
	NM_005178	Bcl3	Bcl3 2	GAGCCUACUGCCUUGUA	147
	NM_006218	PI3K Ca	PI3K Ca 4	CUGAAGAAAGCAUUGACUA	148
	NM_005178	Bcl3	Bcl3 3	GGCCGGAGGCGCUUUAUA	149
	NM_002745	MAPK1	MAPK1 4	GUACAGGGCUCCAGAAAUU	150
	NM_005178	Bcl3	Bcl3 4	UCGACGCAGUGGACAUUAA	151
	NM_006218	PI3K Ca	PI3K Ca 2	AACUAGAAGUAUGUUGCUA	152
	NM_002746	MAPK3	MAPK3 1	GACCGGAUGUUAACCUUUA	153
	NM_000348	SRD5A2	SRD5A2 4	UUGGGUGUCUUCUUAUUUA	154
	NM_006218	PI3K Ca	PI3K Ca 3	AAUGGCUUUGAAUCUUGG	155



**TABLE I**  
**SEQUENCES USED FOR THE DATA OF FIGURES 12-14**

FIG.	ACCESSION #	GENE NAME	DESIGNATION	SIRNA TARGET SEQUENCE	SEQ. ID NO.
	NM_002755	MAP2K1	MAP2K1 1	GCACAUGGAUGGAGGUUCU	156
	NM_006219	PI3K Cb	PI3K Cb 3	GGAUUCAGUUGGAGUGAUU	157
	NM_001654	ARaf1	Raf1 4	CAAAGAACAUCAUCCAUAUAG	158
	NM_001047	SRD5A1	SRD5A1 3	GAAAGCCUAUGCCACUGUU	159
	NM_000348	SRD5A2	SRD5A2 2	GCUAUGCCCUGGCCACUUG	160
	NM_002755	MAP2K1	MAP2K1 4	GAGCAGAUUUUGAAGCAACU	161
	NM_001047	SRD5A1	SRD5A1 2	UAACUGCAGCCAACUAUUU	162
	NM_006218	PI3K Ca	PI3K Ca-1	AUGUUUACUACCAAUAUGGA	163
	NM_030662	MAP2K2	MAP2K2 3	UCCAGGAGUUUGUCAUAUA	164
	NM_001047	SRD5A1	SRD5A1-1	GCAGAUACUUGAGCCAUAUG	165
	NM_002746	MAPK3	MAPK3 3	GAAACUACCUACAGUCUCU	166
	NM_001047	SRD5A1	SRD5A1 4	CCGGAAAUUUGAAGAGUAU	167
	NM_005178	Bcl3	Bcl3 1	GAACACCGAGUGCCAAGAA	168
	NM_000348	SRD5A2	SRD5A2 3	GGACAUUUGUGUACUCACU	169
<b>12 C</b>					
	NM_002755	MAP2K1	MAP2K1 1	GCACAUGGAUGGAGGUUCU	170
	NM_002755	MAP2K1	MAP2K1 2	GCAGAGAGAGCAGAUUUGA	171
	NM_002755	MAP2K1	MAP2K1 3	GAGGUUCUCUGGAUCAAGU	172
	NM_002755	MAP2K1	MAP2K1 4	GAGCAGAUUUUGAAGCAACU	173
	NM_030662	MAP2K2	MAP2K2 1	CAAAGACGAUGACUUCGAA	174
	NM_030662	MAP2K2	MAP2K2 2	GAUCAGCAUUUGCAUGGAA	175
	NM_030662	MAP2K2	MAP2K2 3	UCCAGGAGUUUGUCAUAUA	176
	NM_030662	MAP2K2	MAP2K2 4	GGAAGCUGAUCCACCUUGA	177
<b>12 D</b>					
	NM_030662	MAP2K2	MAP2K2 3	UCCAGGAGUUUGUCAUAUA	178
	NM_001047	SRD5A1	SRD5A1 1	GCAGAUACUUGAGCCAUAUG	179
	NM_001047	SRD5A1	SRD5A1 3	CCGGAAAUUUGAAGAGUAU	180
	NM_000348	SRD5A2	SRD5A2 3	GGACAUUUGUGUACUCACU	181
<b>13A</b>					
	NM_005990	STK10		GAAACGAGAUUCCUUAUC	182
	AY406545	MADH6		CAAGAUCGGUUUUGGCAUA	183
	NM_170679	SKP1A		CAAACAAUCUGUGACUAUU	184
	NM_002257	KLK1		CAACUUGUUUGACGACGAA	185
	NM_000942	PPIB		GAAAGGAUUUGGCUACAAA	186
	NM_005083	U2AF1L1		GAGCAUGUUUACAACGUUU	187
	NM_000942	PPIB		GGAAAGACUGUUCCAAAAA	188
	NM_006622	SNK		ACAUUUACAUCUCUUGGA	189
	NM_000942	PPIB		GAAAGAGCAUCUACGGUGA	190
	NM_005379	MYO1A		ACAAGGAGAUUUUAUACCUA	191
	NM_002620	PF4V1		AGGAACAUUUGGAGAGUUA	192
	NM_005627	Sgk1		CAUCGUUUUAUAGAGACUUA	193

**TABLE I**  
**SEQUENCES USED FOR THE DATA OF FIGURES 12-14**

FIG.	ACCESSION #	GENE NAME	DESIGNATION	siRNA TARGET SEQUENCE	SEQ. ID NO.
	NM_022550	XRCC4		GAAAGUAAGCAGAAUCUAU	194
	AY313906	SARS SEP		AACCAACGGUUUACGUCUA	195
	NM_181523	PIK3R1		GAAAGACAAGAGACCAAUA	196
	NM_020183	ARNTL2		CAACAGCGAUUUUAGGAUA	197
	NM_018131	C10ORF3		GGAAACAGCUGCUCAUUA	198
	NM_139025	ADAMTS13		ACAUUUGGCUGUGAUGGUA	199
	NM_005767	P2RY5		GAAACUACAACUUACAUGA	200
	NM_147199	MRGX1		GAUGAUGUUUCCUACUUU	201
	NM_001892	CSNK1A1		AGAAUUUGCGAUGUACUUA	202
	NM_006930	SKP1A		AGGUUUGCUUGAUGUUACA	203
	M15077	PPYLUC		CGAAAGGUCUUACCGGAA	204
	NM_006257	PRKCQ		CAAAGAGUAUGUCGAAUCA	205
	NM_018131	C10ORF3		AAGGAAAGCUGACUGAUAA	206
	NM_013391	DMGDH		CAUCAAGCUGCCAUGGAA	207
	BC025733	FADD		CAGCAUUUAACGUCAUAUG	208
	NM_005541	INPP5D		AUUGCGUUUACACUUACAG	209
	NM_006395	GSA7		GAUCAAGGUUUUCACUAA	210
	AC146999	Human Herpes- virus 5		CAAACCAGCGCGCUAAUGA	211
	NM_153202	ADAM33		CAAACAGCGUCUCCUGGAA	212
	NM_005508	CCR4		GAAAGCAUAUACAGCAAUU	213
	NM_002605	PDE8A		CAAAGAAGAUAAACCAUGU	214
	NM_000455	STK11		GAAACAUCCUCCGGCUGAA	215
	AF493910	RALA		GAGCAGAUUUUAAGAGUAA	216
	NM_012184	FOXD4L1		GGACAAUUUUGCAGCAACA	217
	NM_001273	CHD4		CAAAGGUGCUGCUGAUGUA	218
	NM_002434	MPG		ACAUCAUUUACGGCAUGUA	219
<b>13B</b>					
	NM_004429	EFNB1		CCACACCGCUGGCCAAGAA	220
	NM_002717	PPP2R2A		UAUCAAGCCUGCCAUAUG	221
	XM_110671	M11		UCAUAAGCCAUCUUCUAA	222
	NM_001282	AP2B1		GAGCUAAUCUGCCACAUG	223
	NM_001846	COL4A2		CGAAGGCGGUGGCCAAUCA	224
	AF100153	CNK		GCACAUCCGUUGGCCAUCA	225
	NM_001136	AGER		GCCAGGCAAUGAACAGGAA	226
	NM_007122	USF1		GGAAGCCAGCGCUCAAUG	227
	NM_001136	AGER		GCGAGCCACUGGUGCUGAA	228
	NM_018653	GPRC5C		CCACCUCGUGGCCAUAUG	229
	NM_001431	EPB41L2		GAAGGACUCUAGCCAGUUA	230
	NM_000119	EPB42		GACCACACCUUGCCAUCAA	231
	NM_004448	ERBB2		GCAGUUACCAGUGCCAAUA	232
	NM_005971	FXVD3		GGACGCCAAUGACCUAGAA	233
	NM_003494	DYSF		GAACUAUGCUGCCAUGAAG	234
	NM_013391	DMGDH		CAUCAAGCUGCCAUGGAA	235

**TABLE I**  
**SEQUENCES USED FOR THE DATA OF FIGURES 12-14**

FIG.	ACCESSION #	GENE NAME	DESIGNATION	siRNA TARGET SEQUENCE	SEQ. ID NO.
	NM_022353	OSGEPL1		AGACAUUGCUGCCACAGUA	236
	NM_003367	USF2		GGCCAGUUCUACGUCAUGA	237
	NM_172390	NFATc1		GCCAGGAGCUGAACAUUAA	238
<b>13C</b>					
	NM_005378	MYCN		CACGUCCGCUCAAGAGUGU	239
	NM_000147	FUCA1		UAACAAUGCUGGGAAUUCA	240
	NM_003566	EEA1		AGACAGAGCUUGAGAAUAA	241
	NM_004707	APG12L		UGUUGCAGCUUCCUACUUC	242
	NM_003918	GYG2		GACCAAGGCUUACUGAAUA	243
	NM_004462	FDFT1		CAUAGUUGGUGAAGACAUUA	244
	XM_291277	SgK223		GAGCUCCACUUCAAUGAGA	245
	NM_004573	PLC beta 2		GAACAGAAGUUACGUUGUC	246
	NM_003955	SOCS3		CACCUGGACUCCUAUGAGA	247
	NM_203330	CD59		CUACAACUGUCCUAACCCA	248
	NM_002377	MAS1		CUACACAAUUGUCACAUUA	249
	NM_153326	AKR1A1		UGAGGAGGCUGAGUAAUUC	250
	NM_001749	CAPNS1		CCACAGAACUCAUGAACAU	251
	NM_016735	LIMK1		UCAACUUCAUCACUGAGUA	252
	NM_002393	MDM4		CGUCAGAGCUUCUCCGUAA	253
	NM_021969	NR0B2		CGUAGCCGCUGCCUAUGUA	254
	NM_002741	PRKCL1		ACAGCGACGUGUUCUCUGA	255
	NM_014452	TNFRSF21		CAGAAGGCCUCGAAUCUCA	256
	NM_139343	BIN1		GCUCAAGGCUGGUGAUGUG	257
	NM_001003945	ALAD		GAUGACAUACAGCCUAUCA	258
	NM_013315	TPTE		UUUAUUCGAUUCCUCGUUA	259
	NM_024560	FLJ21963		UCGAGUGGAUGAUGUAAUA	260
	L07868	ERBB4		AGGAUCUGCAUAGAGUCUU	261
	NM_001003809	DLGAP1		CAACCUGGAUGGUGACAUG	262
	NM_005232	EPHA1		UGAAGAACGGUACCAGAUG	263
	NM_003818	CDS2		GUGAGACAGUGACGGAUUA	264
	NM_153675	FOXA2		ACGAACAGGUGAUGCACUA	265
	XM_496495	GGT2		AAUAAUGAAUGGACGACUU	266
	NM_020676	ABHD6		GAUGACCUGUCCAUAAGAUG	267
	NM_000487	ARSA		UCUAUGACCUGUCCAAGGA	268
	AF348074	NAT2		AUACAGAUCUGGUCGAGUU	269
	U02388	CYP4F2		CAUAUUGACUCCUGUAUU	270
<b>14 A- 14 I</b>					
		EGFP		GCAAAGACCCCAACGAGAA	271
	NM_012154	eIF2C2		GCACGGAAGUCCAUCUGAA	272
				GCAGGACAAAGAUGUAUUA	273
				GGGUCUGUGGUGAUAAUUA	274
				GUAUGAGAACCCAAUGUCA	275

TABLE I					
SEQUENCES USED FOR THE DATA OF FIGURES 12-14					
FIG.	ACCESSION #	GENE NAME	DESIGNATION	siRNA TARGET SEQUENCE	SEQ. ID NO.
	NM_012154	eIF2C2		GCACGGAAGUCCAUCUGAA	276
				GCAGGACAAAGAUGUAUUA	277
				GGGUCUGUGGUGAUAAAUA	278
				GUAUGAGAACCCAAUGUCA	279
<b>14J</b>	NM_006218	PI3K Ca	PI3K Ca-1	AUGUUUACUACCAAUGGA	280
	NM_001047	SRD5A1	SRD5A1 2	UAACUGCAGCCAACUAUUU	281
	NM_030662	MAP2K2	MAP2K2 3	UCCAGGAGUUUGUCAUAA	282
	NM_001047	SRD5A1	SRD5A1-1	GCAGAUACUUGAGCCAUG	283
	NM_001047	SRD5A1	SRD5A1 4	CCGGAUUUGAAGAGUAU	284
<b>14K</b>					
	NM_006218	PI3K Ca	PI3K Ca 1	AUGUUUACUACCAAUGGA	285
	NM_001047	SRD5A1	SRD5A1 2	UAACUGCAGCCAACUAUUU	286
	NM_030662	MAP2K2	MAP2K2 3	UCCAGGAGUUUGUCAUAA	287
	NM_001047	SRD5A1	SRD5A1 1	GCAGAUACUUGAGCCAUG	288
	NM_001047	SRD5A1	SRD5A1 4	CCGGAUUUGAAGAGUAU	289
	NM_000348	SRD5A2	SRD5A2 3	GGACAUUUGUGUACUCACU	290
	M15077	PPYLUC		UGUUUGUGGACGAAGUACC	291
	BC020308	GAPDH		CCUGGCCAAGGUCAUCCA	292
	NM_000942	PPIB		GAGAAAGGAUUUGGCUACA	293
<b>14L</b>					
	NM_001047	SRD5A1	SRD5A1 2	UAACUGCAGCCAACUAUUU	294
	NM_030662	MAP2K2	MAP2K2 3	UCCAGGAGUUUGUCAUAA	295
	NM_001047	SRD5A1	SRD5A1 1	GCAGAUACUUGAGCCAUG	296
	NM_001047	SRD5A1	SRD5A1 4	CCGGAUUUGAAGAGUAU	297
	NM_000348	SRD5A2	SRD5A2 3	GGACAUUUGUGUACUCACU	298
	NM_001273	CHD4		CAAAGGUGCUGCUGAUGUA	299
	NM_002605	PDE8A		CAAAGAAGAUAAACCAAUGU	300
	NM_000455	STK11		GAAACAUCCUCCGGCUGAA	301

Note: Sequences for 14L are also present in a modified form where positions 1 and 2 of the sense and antisense strands contain 2'-O-methyl groups and positions 1 of the antisense strand also contains a phosphate group on the 5' carbon.

5

The data above support a hypothesis that siRNA can induce toxicity in a sequence specific, target independent mechanism. The inventors performed two separate experiments to test the dependence of siRNA-induced toxicity on the RNAi mechanism. The results of these experiments are presented below and demonstrate

that the toxicity is mediated by the RNAi pathway. As previous experiments demonstrated that the toxicity was not associated with target knockdown, a third experiment was performed to determine whether the chemical modification patterns described in the first embodiment could eliminate the toxicity.

5

In the first experiment, the ability of toxic motif containing siRNA to induce cell death was investigated under circumstances where the RNAi mechanism was severely compromised. Previous studies revealed that eIF2C2/hAgo2 is responsible for mRNA cleavage and that knockdown of this gene product severely cripples this pathway. The inventors confirmed this finding (**Figure 14 a-i**) and then assessed the importance of this pathway in newly discovered siRNA-induced toxicity. To test this, cells transfected with the eIF2C2/hAgo2 siRNA pool (T1) were subsequently transfected with toxic siRNA containing either the AAA/UUU or GCCA/UGG motifs (**Figure 14a**, “Experiment”). The results of these experiments demonstrated that in the absence of an intact RNAi pathway, toxic siRNA were unable to induce the cell death phenotype (**Figure 14j**). As parallel experiments where the RNAi pathway was left intact exhibited toxicity characteristic of these sequences, it was concluded that an intact RNAi pathway was necessary for siRNA-induced toxicity. These experiment strongly support the hypothesis that toxic siRNA induce their phenotype through the RNAi pathway. Since the observed toxicity is unrelated to the level or degree of target knockdown, it is likely that off-targeting is responsible for the observed toxic phenotype.

Further support for the involvement of the RNAi pathway in siRNA toxicity came from an experiments where the size of the duplex was reduced from 19bp to 17 bp. Previous studies have shown that duplexes that are shorter than 19bp targeted mRNA sequences inefficiently, most likely due to the fact that Dicer and/or RISC fail to mediate RNAi when duplex sequence length drops below 19bp (Elbashir, S.M. *et al.* “Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells” *Nature* 2001 May 24;411(6836):494-8). When the length of known, 19bp, toxic siRNA was reduced by 2 bp (17 bp total length, no disruption of the motif) the level of toxicity was reduced dramatically (**Figure 14k**), suggesting that entry and/or processing by RISC is necessary for induction of toxicity. These results again implicate the RNAi pathway in this form of siRNA induced cellular toxicity.

Since the observed toxicity is unrelated to the level or degree of target knockdown, it is likely that off-targeting is responsible for the observed toxic phenotype.

As noted above, the modifications described in the first embodiment of the invention have been shown to eliminate off-target effects. As siRNA-induced cellular toxicity is dependent on RNAi, but unrelated to target knockdown, the inventors decided to test whether modifications that eliminate off-targeting, abolish siRNA-induced cellular toxicity. To accomplish this, a variation of the chemical modification pattern described in the first embodiment was added to siRNA that were known to induce toxicity in an RNAi- dependent mechanism. Specifically, siRNA synthesized to carry the following modifications: 2'-O-methyl groups on positions 1 and 2 of both the sense and antisense strands, plus a 5' phosphate group on carbon 5 of the 5' terminal antisense nucleotide. As shown in **Figure 14-I**, when eight separate unmodified, toxic siRNA (MAP2K2 d3, SRD5A1 d1, SRD5A1 d2, SRD5A1 d4, SRD5A2 d3, PDE8A, STK11, and CHD4) were transfected into cells, each decreased cell viability below 75%. In contrast, chemical modification of all eight duplexes markedly decreased siRNA-induced toxicity without significantly altering target specific knockdown. These findings strongly support the premise that siRNA induced toxicity induced by AAA/UUU or GCCA/UGGC containing siRNA is the result of off-target effects. More importantly, the findings presented here suggest that off-target induced phenotypes can be eliminated by the addition of the modifications of the invention.

These studies were extended by assessing the effects of modification patterns of the invention on an additional thirty-seven siRNA that targeted luciferase (Accession No. M15077). Specifically, the sequences listed in **Table II** below were synthesized in both modified (2'-O-methyl on positions 1 and 2 of the sense strand, 2'-O-methyl on position 2 of the antisense strand, phosphate on the 5' carbon of the 5' terminal nucleotide of the antisense strand) and unmodified states, and transfected into HeLa cells (5,000 cells per well, 10uM siRNA, 0.1 microliter Lipofectamine 2000 per well). Subsequently, the level of cell death in each culture was measured 72 hours after transfection using Alamar Blue.

**Table II** lists toxic luc sequences used in this study and includes (1) the SEQ. ID NO. (right column) and (2) the sequence (left column, 5'→3', sense strand).

<b>TABLE II</b> <b>LUC WALK – TOXIC SEQUENCES</b>	
<b>SEQUENCE</b>	<b>SEQ. ID NO.</b>
AGAUCCUCAUAAAGGCCAA	302
AGAGAUCCUCAUAAAGGCC	303
AAUCAGAGAGAUCCUCAUA	304
AAAAUCAGAGAGAUCCUCA	305
GAAAAAUCAGAGAGAUCCU	306
GCAAGAAAAAUCAGAGAGA	307
CUCGACGCAAGAAAAAUCA	309
GGAAAACUCGACGCAAGAA	309
CUUACCGGAAAACUCGACG	310
GUCUUACCGGAAAACUCGA	311
AGGUCUUACCGGAAAACUC	312
CGAAAGGUCUUACCGGAAA	313
AAGUACCGAAAGGUCUUAC	314
UGGACGAAGUACCGAAAGG	315
UGUUUGUGGACGAAGUACC	316
UGUGUUUGUGGACGAAGUA	317
GUUGUGUUUGUGGACGAAG	318
UUGCGCGGAGGAGUUGUGU	319
AAAGUUGCGCGGAGGAGUU	320
AGUCAAGUAACAACCGCGA	321
GUCGCCAGUCAAGUAACAA	322
GAUUACGUCGCCAGUCAAG	323
UGGAUUACGUCGCCAGUCA	324
CGUGGAUUACGUCGCCAGU	325
AGAUCGUGGAUUACGUCGC	326
AGAGAUUGUGGAUUACGUC	327
AAAAAGAGAUUGUGGAUUA	328
ACGGAAAAAGAGAUUGUGG	329
UGACGGAAAAAGAGAUUGU	330
GAGCACGGAAAGACGAUGA	331
UGGAGCACGGAAAGACGAU	332

TABLE II LUC WALK – TOXIC SEQUENCES	
SEQUENCE	SEQ. ID NO.
UUUGGAGCACGGAAAGACG	333
GUUUUGGAGCACGGAAAGA	334
UGUUGUUUUGGAGCACGGA	335
CCGUUGUUGUUUUGGAGCA	336
AACUUCCCGCCGCGGUUGU	337
UGAACUUCCCGCCGCGGUU	338

The results of these studies are presented in **Figure 14m** and show (1) in twenty-five out of thirty-seven cases, the level of toxicity dropped significantly upon addition of chemical modifications of the invention, and (2) in twenty-two out of the twenty-cases, the level of toxicity drop below the 25% level previously set as the line discriminating between toxic and non-toxic transfections. As additional studies designed to compare the functionality of modified and unmodified luc sequences showed comparable activity in both sets of molecules (**Figure 14n**, HEK 293 cells, 20,000 cells per well, 0.3 microliters of Lipofectamine 2000 per well, 72 hour time point, 100nM siRNA) these findings strongly support the premise that (1) siRNA induced toxicity brought about by AAA/UUU or GCCA/UGGC containing siRNA is the result of off-target effects, and (2) that off-target induced phenotypes can be greatly diminished or eliminated by the addition of the modifications of the invention.

#### EXAMPLE 11

##### THE SYNERGISTIC EFFECTS OF CHEMICAL MODIFICATIONS OF THE INVENTION AND POOLING ON ELIMINATING OFF-TARGET EFFECTS

To assess the value of combining chemical modifications of the invention and pooling to eliminate off-target silencing, four siRNA as well as the respective pool of molecules, targeting human cyclophilin B (PPIB) were tested for off-target effects (in both modified and unmodified forms) using microarray-based gene expression profiling. To accomplish this, the sequences targeting PPIB (see below) were first synthesized in unmodified and modified (2'-O-methyl on positions 1 and 2 of the sense strand, 2'-O-methyl on position 2 of the antisense strand, phosphate on the 5' carbon of the 5' terminal nucleotide of the antisense strand) forms.



**Sequences: Sense Strand**

PPIB-1/C1: 5'-GAAAGAGCAUCUACGGUGA-3' (SEQ. ID NO. 339)

PPIB -2/C2: 5'-GAAAGGAUUUGGCUACAAA-3' (SEQ. ID NO. 340)

PPIB -3/C3: 5'-ACAGCAAAUCCAUCGUGU-3' (SEQ. ID NO. 341)

5 PPIB -4/C4: 5'-GGAAAGACUGUCCAAAAA-3' (SEQ. ID NO. 342)

Subsequently, duplexes were transfected into human HeLa cells (10K cells per well of a 96 well plate, 0.4 uL Dharmafect 1 lipid per well (Dharmacon, Inc.), 100nM concentration. Note, in the case of the pool, the concentration of each duplex was 25  
10 nM, thus the total concentration remained consistent with studies performed on individual siRNA). Cell lysates for microarray analysis were then collected (24 hours after transfection) and total RNA purification was performed using Qiagen's RNeasy columns with on-column DNase digestion. RNA integrity was analyzed with the  
15 RNA 6000 Nano LabChip on Agilent's 2100 Bioanalyzer. To perform gene expression profiling the following procedures were performed: for each sample, 650 ng of total RNA was amplified and Cy3- or Cy5-labeled (Perkin Elmer) using Agilent's Low Input RNA Fluorescent Linear Amplification Kit. Hybridizations were performed using Agilent's Human 1A (V2) Oligo Microarrays  
(<http://www.agilent.com>). The hybridization reference (Cy3) was mock-transfected  
20 cells or untransfected cells, as indicated. Slides were washed and dried using 6X and 0.06X SSPE with 0.025% N-lauroylsarcosine, pure acetonitrile, and Agilent's proprietary non-aqueous drying and stabilization solution. Subsequently, they were scanned on an Agilent Microarray Scanner (model G2505B) and the raw image was processed using Feature Extraction (v7.1.1.). Further analysis/data processing was  
25 conducted using Spotfire Decision Site 8.0 and the Spotfire Functional Genomics Module. Low signal genes were removed from the analysis by applying a cutoff of the Log(red and green additive signal in pixels)>2.8 from the data on a representative self-self array. Removal of feature outliers was not conducted. A 2-fold cutoff (Log Ratio of >0.3 or <-0.3) was applied to genes used in comparative analysis.

30

Results from the analysis of cyclophilin B targeting siRNA showed that while the level of targeted gene knockdown remained constant in modified and unmodified duplexes (data not shown), in all cases the addition of the chemical modification pattern of the invention reduced the number of off-targeted genes (down regulated by

more than two fold) by 50% or more. For duplexes C1, C2, C3, and C4, the number of off-targets were reduced from 5→2, 57→23, 12→6, and 72→21, respectively (Note, in the case of C2, 17 of the 23 off-targets are believed to be experimental artifacts, thus the reduction in this case may be as great as 57→6). These results support  
5 previous findings (see **Figures 7-11**) that show addition of modifications of the invention strongly suppress off-target effects. **Figure 15c** shows that the number of off-targets generated by the unmodified pool is less than that which would be predicted from the four individual siRNA (*i.e.* the sum of off-targets generated by each duplex = 5 + 57 + 12 + 72 = 146 vs. the pool of all four duplexes = 65). Thus,  
10 in this case, pooling by itself can reduce the number of off-targets by approximately 55%. To the surprise of the inventors, only four off-targets were identified using modified pools. Compared to the total number of off-targets observed in unmodified, individual siRNA (146), this represents a 97.2% reduction in the total number of off-targets. Compared to the number of off-targets observed in unmodified pools (65),  
15 this change (65→4) represents a net reduction of 94%. These unexpected and striking results demonstrate that combining the modifications of the invention with pooling, provides a striking dramatic advantage over individual siRNA or pools of siRNA.

To determine whether the observed benefits of combining chemical modifications  
20 with pooling were confined to sequences targeting cyclophilin B, siRNA targeting a second gene (human mek1, MAP2K1, Accession No. NM\_002755) were tested in a identical format. The sequences used in this study are shown below:

MAP2K1-1 (M1): 5'-GCACAUGGAUGGAGGUUCU-3' (**SEQ. ID NO. 343**)  
25 MAP2K1-2 (M2): 5'-GCAGAGAGAGCAGAUUUGA-3' (**SEQ. ID NO. 344**)  
MAP2K1-4 (M4): 5'-GAGCAGAUUUGAAGCAACU-3' (**SEQ. ID NO. 345**)  
MAP2K1-5 (M5): 5'-CCAGAAAGCUAAUUCAUCU-3' (**SEQ. ID NO. 346**)

As was the case with cyclophilin B, addition of the chemical modification of  
30 the invention to MAP2K1 targeting siRNA did not appreciably affect targeted gene knockdown. But as previously observed, addition of chemical modifications of the invention to individual siRNA targeting MAP2K1 reduced (in all cases with the exception of one that is suspected to be an artifact, *i.e.* M4) the number of genes that were off-targeted by two fold (or more) by 50% or greater (85→8, 9→2, 58→32,

15→41 (artifact), see **Figures 15d-f**. Furthermore, as was observed in previous studies with PPIB, the pooling of individual duplexes generated fewer off-targets than the combined number observed with individual siRNA (pool off-targets = 26 vs. combined individual off-targets = 167). While this represents an 85% reduction in the total number of off-targets, combining chemical modifications with pooling again enhanced the specificity of the pool alone. Only two genes were observed to be down regulated by two-fold or more when pools containing modified MAP2K1 targeting siRNA were used. Compared to the results generated with individual, unmodified siRNA (167) this represents a 98.8% reduction in the number of off-targets.

Compared to unmodified pools (26 total off-targets), the number of off-targets observed in modified pools (2) represents a 92.3% reduction in the total number of off-targeted genes. Together, these data demonstrate that combination of modifications of the invention and pooling provided a previously unobtainable level of gene knockdown specificity. This enhanced specificity is observed regardless of the targeted gene and would greatly reduce the number of false positive in *e.g.* high throughput screens designed to identify potential drug targets.

**WHAT IS CLAIMED IS:**

2. A double stranded ribonucleotide comprising:
- i. a sense strand comprising a sense region, wherein said sense region comprises:
    - 5 i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification, and
    - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
  - 10 ii. an antisense strand comprising an antisense region, wherein said antisense region comprises
    - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated, and
    - 15 ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification,
- 15 wherein said sense region and said antisense region are capable of forming a duplex of 18 –24 base pairs of nucleotides that has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide, said first
- 20 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.
3. The double stranded ribonucleotide of claim 1, wherein said first 5' antisense
- 25 nucleotide comprises an –OH at its 2' position.
4. The double stranded ribonucleotide of claim 2, wherein said first 2'-O-alkyl modification comprises 2'-O-methyl, said second 2'-O-alkyl modification comprises 2'-O-methyl, and said third 2'-O-alkyl modification comprises 2'-O-
- 30 methyl.

5. The double stranded ribonucleotide according to claim 3, further comprising a 2'-OH on all nucleotides other than said first 5' sense nucleotide, said second 5' sense nucleotide and said second 5' antisense nucleotide.
- 5 6. The double stranded ribonucleotide of claim 1, wherein said first 5' antisense nucleotide comprises a fourth 2'-O-alkyl modification.
7. The double stranded ribonucleotide of claim 5, wherein said first 2'-O-alkyl modification comprises 2'-O-methyl, said second 2'-O-alkyl modification  
10 comprises 2'-O-methyl, said third 2'-O-alkyl modification comprises 2'-O-methyl, and said fourth 2'-O-alkyl modification comprises 2'-O-methyl.
8. The double stranded ribonucleotide according to claim 6, further comprising a 2'-OH on all nucleotides other than on said first 5' sense nucleotide, said second 5'  
15 sense nucleotide, said first 5' antisense nucleotide, and said second 5' antisense nucleotide.
9. The double stranded ribonucleotide of claim 1, further comprising a 3' overhang of 1 to 6 bases on at least one of said sense strand and said antisense strand.  
20
10. The double stranded ribonucleotide of claim 4, further comprising a 3' overhang of 1 to 6 bases on at least one of said sense strand and said antisense strand.
11. The double stranded ribonucleotide of claim 7, further comprising a 3' overhang  
25 of 1 to 6 bases on at least one of said sense strand and said antisense strand.
12. A kit, comprising at least two siRNA, wherein the at least two siRNA comprise a first siRNA and a second siRNA, and wherein each of the first siRNA and the second siRNA comprises:  
30
  - i. a sense strand, wherein said sense strand comprises
    - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and
    - ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and

- ii. an antisense strand, wherein said antisense strand comprises
    - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
    - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;
- wherein said sense strand and said antisense strand are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.
13. A kit according to claim 11, wherein the first siRNA and the second siRNA each comprises a sequence that is at least substantially complementary to a region in a target mRNA.
14. A kit according to claim 12, wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.
15. A kit according to claim 12, wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.
16. A kit, comprising at least two unimolecular siRNA, wherein the at least two siRNA comprise a first siRNA and a second siRNA, and wherein each of the first siRNA and the second siRNA comprises:
- i. a sense region, wherein said sense region comprises
    - i. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and

- ii. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and
  - ii. an antisense region, wherein said antisense region comprises
    - i. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and
    - ii. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;
- wherein said sense region and said antisense region are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense region, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

17. A kit according to claim 15, wherein the first siRNA and the second siRNA each comprises a sequence that is at least substantially complementary to a region in a target mRNA.

18. A kit according to claim 16, wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.

19. A kit according to claim 17, wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.

20. A method for minimizing off-target effects in RNAi, said method comprising:

- i. exposing at least two unimolecular siRNA to a target nucleic acid or to a cell, wherein the at least two unimolecular siRNA comprise a first unimolecular siRNA and a second unimolecular siRNA, wherein each of

the first unimolecular siRNA and the second unimolecular siRNA comprises

i. a sense region, wherein said sense region comprises

1. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and

2. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and

ii. an antisense region, wherein said antisense region comprises

1. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and

2. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;

wherein said sense region and said antisense region are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense region, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense region and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

21. A method according to claim 19, wherein the first unimolecular siRNA and the second unimolecular siRNA each comprises a sequence that is at least substantially complementary to a region in a target mRNA.

22. A method according to claim 20, wherein the region of the target mRNA to which the sequence of the first unimolecular siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second unimolecular siRNA is at least substantially complementary.



23. A method according to claim 20, wherein the region of the target mRNA to which the sequence of the first unimolecular siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second unimolecular siRNA is at least substantially complementary.

24. A method for minimizing off-target effects in RNAi, said method comprising:

i. exposing at least two siRNA to a target nucleic acid or to a cell, wherein the at least two siRNA comprise a first siRNA and a second siRNA, wherein each of the first siRNA and the second siRNA comprises

i. a sense strand, wherein said sense strand comprises

1. a first 5' sense nucleotide, wherein said first 5' sense nucleotide comprises a first 2'-O-alkyl modification; and

2. a second 5' sense nucleotide, wherein said second 5' sense nucleotide comprises a second 2'-O-alkyl modification; and

ii. an antisense strand, wherein said antisense strand comprises

1. a first 5' antisense nucleotide, wherein said first 5' antisense nucleotide is phosphorylated; and

2. a second 5' antisense nucleotide, wherein said second 5' antisense nucleotide comprises a third 2'-O-alkyl modification;

wherein said sense strand and said antisense strand are capable of forming a duplex of 18-24 base pairs of nucleotides, wherein the duplex has at least 80% complementarity over the range of the duplex, and within said duplex said first 5' sense nucleotide is the 5' most nucleotide of the sense strand, and said second 5' sense nucleotide is immediately adjacent to and downstream of the first 5' sense nucleotide; said first 5' antisense nucleotide is the 5' most nucleotide of the antisense strand and said second 5' antisense nucleotide is immediately adjacent to and downstream of the first 5' antisense nucleotide.

25. A method according to claim 23, wherein the first siRNA and the second siRNA each comprises a sequence that is at least substantially complementary to a region in a target mRNA.
- 5    26. A method according to claim 24, wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary overlaps the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.
- 10   27. A method according to claim 24, wherein the region of the target mRNA to which the sequence of the first siRNA is at least substantially complementary does not overlap the region of the target mRNA to which the sequence of the second siRNA is at least substantially complementary.

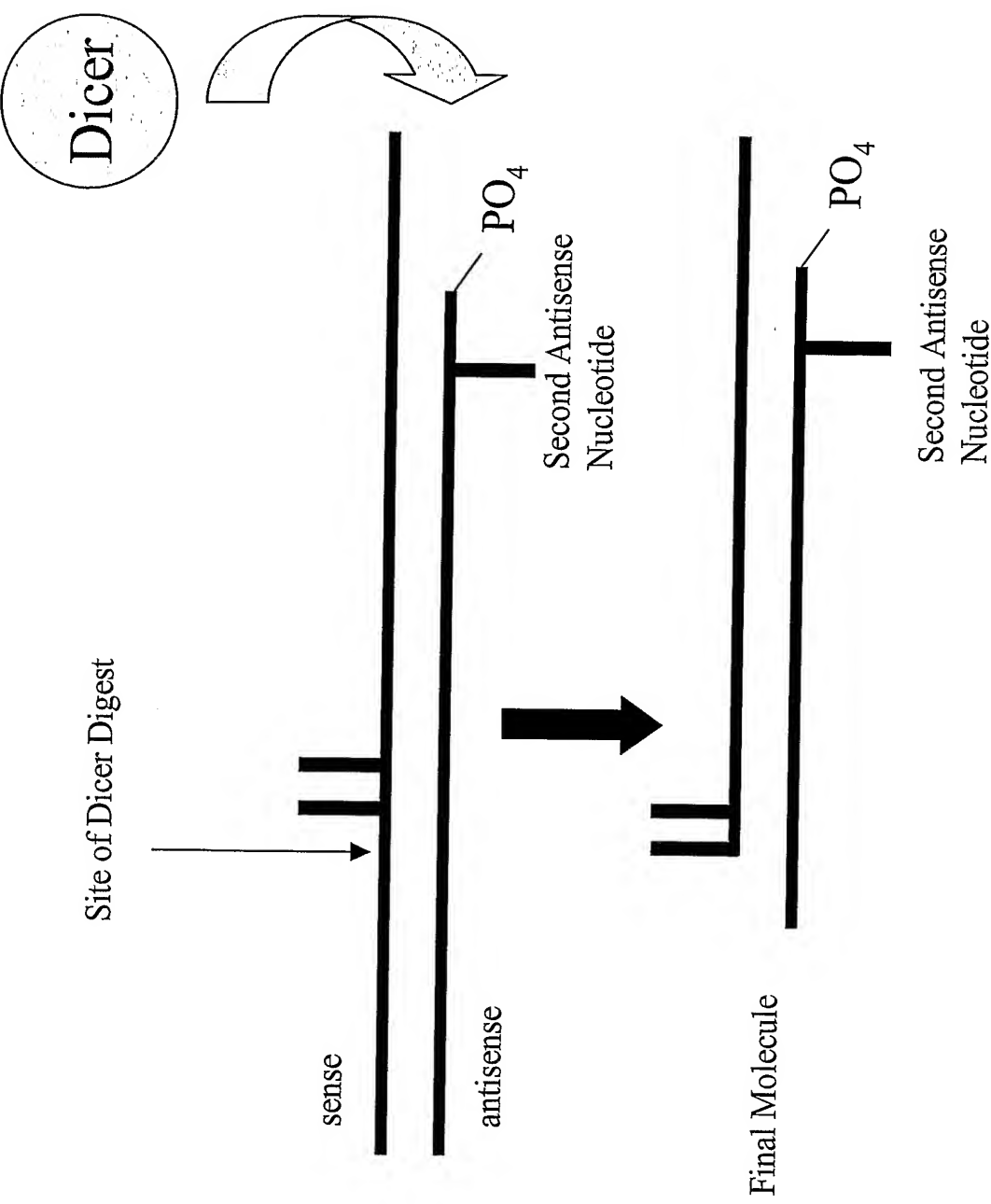


FIG. 1A

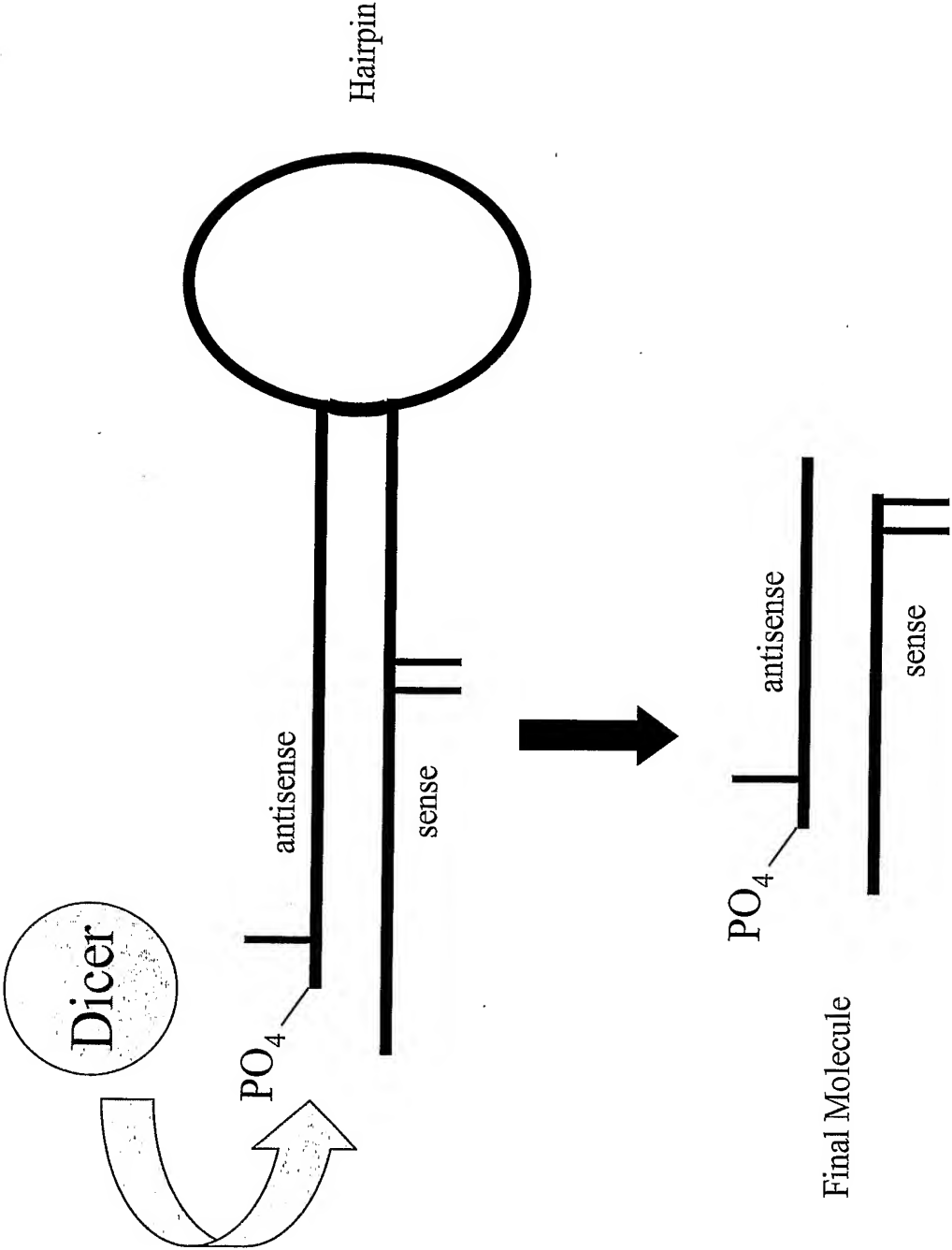
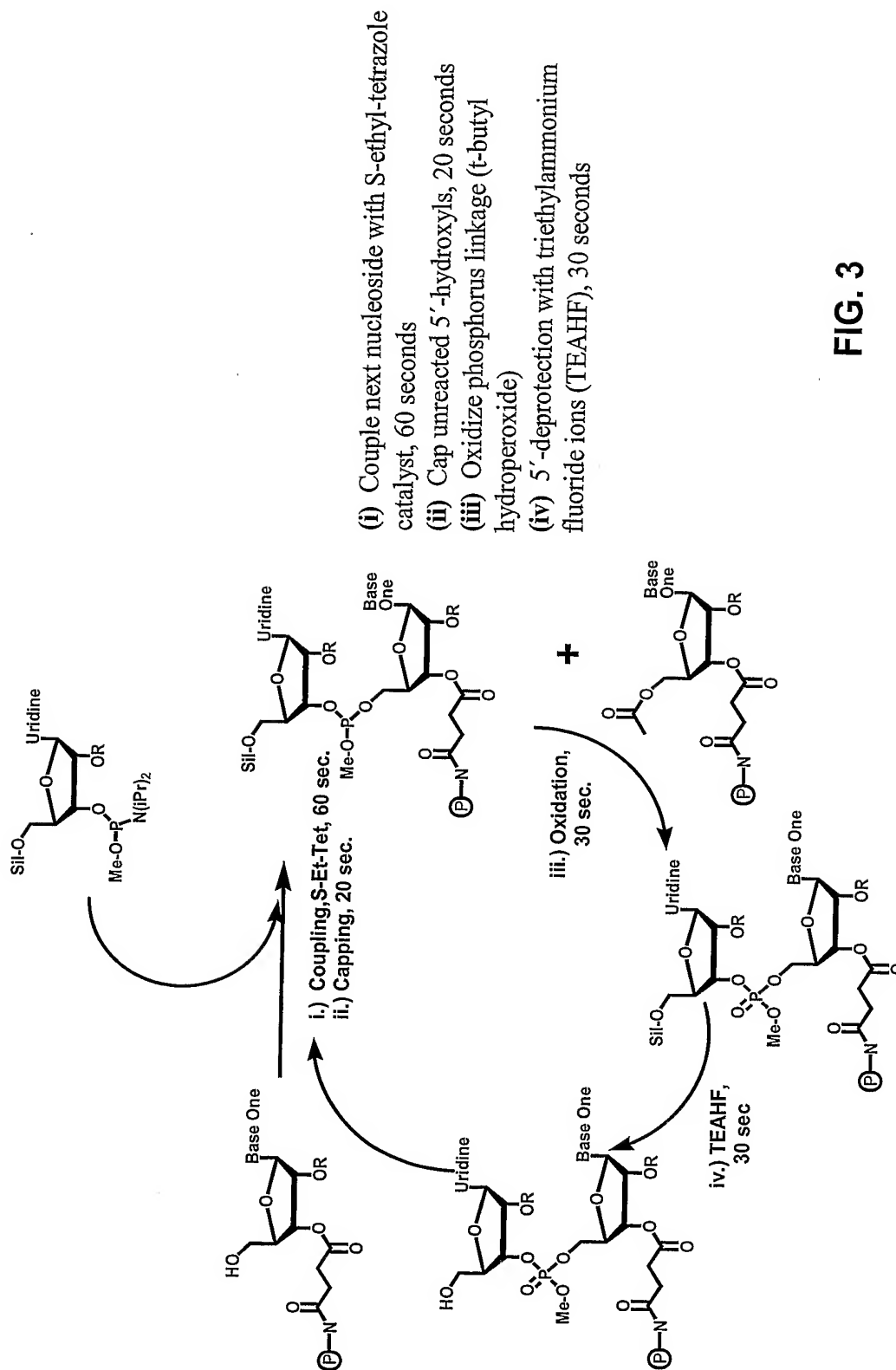


FIG. 2



- (i) Couple next nucleoside with S-ethyl-tetrazole catalyst, 60 seconds
- (ii) Cap unreacted 5'-hydroxyls, 20 seconds
- (iii) Oxidize phosphorus linkage (t-butyl hydroperoxide)
- (iv) 5'-deprotection with triethylammonium fluoride ions (TEAHF), 30 seconds

FIG. 3

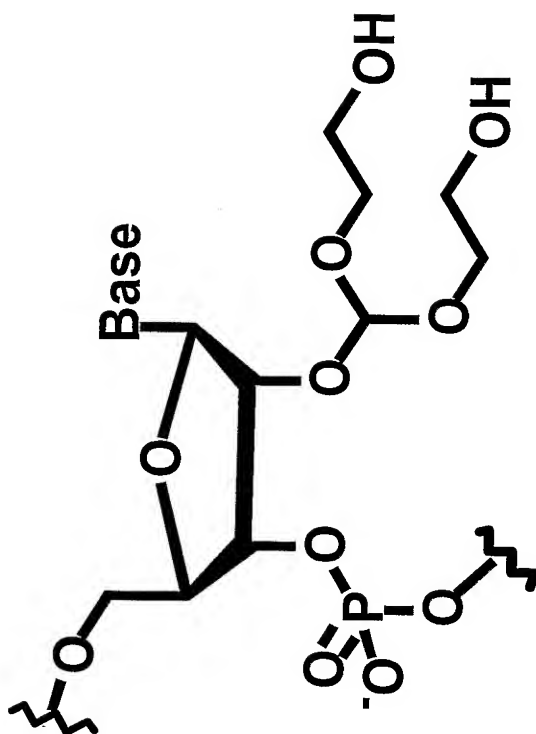


FIG. 4

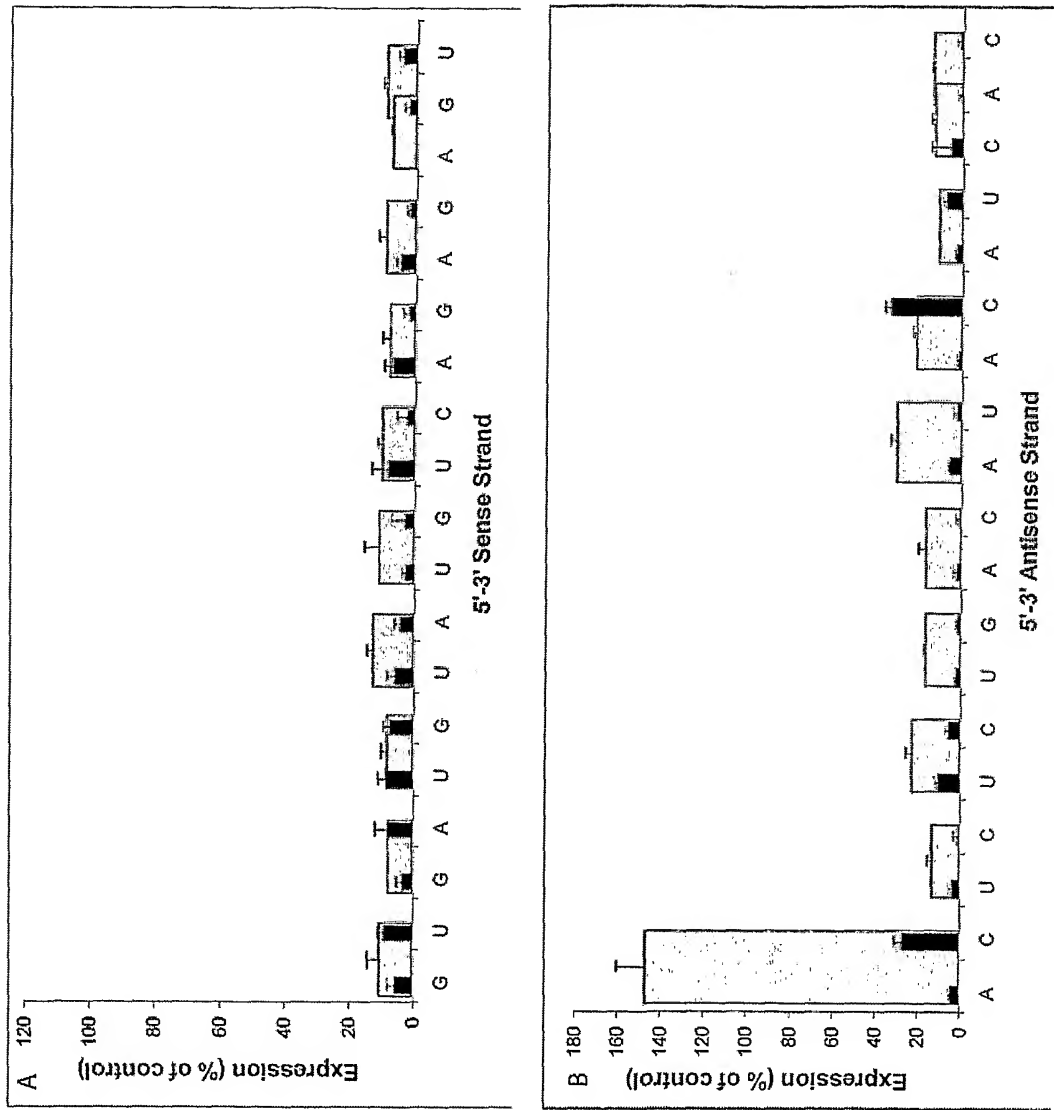


FIG. 5

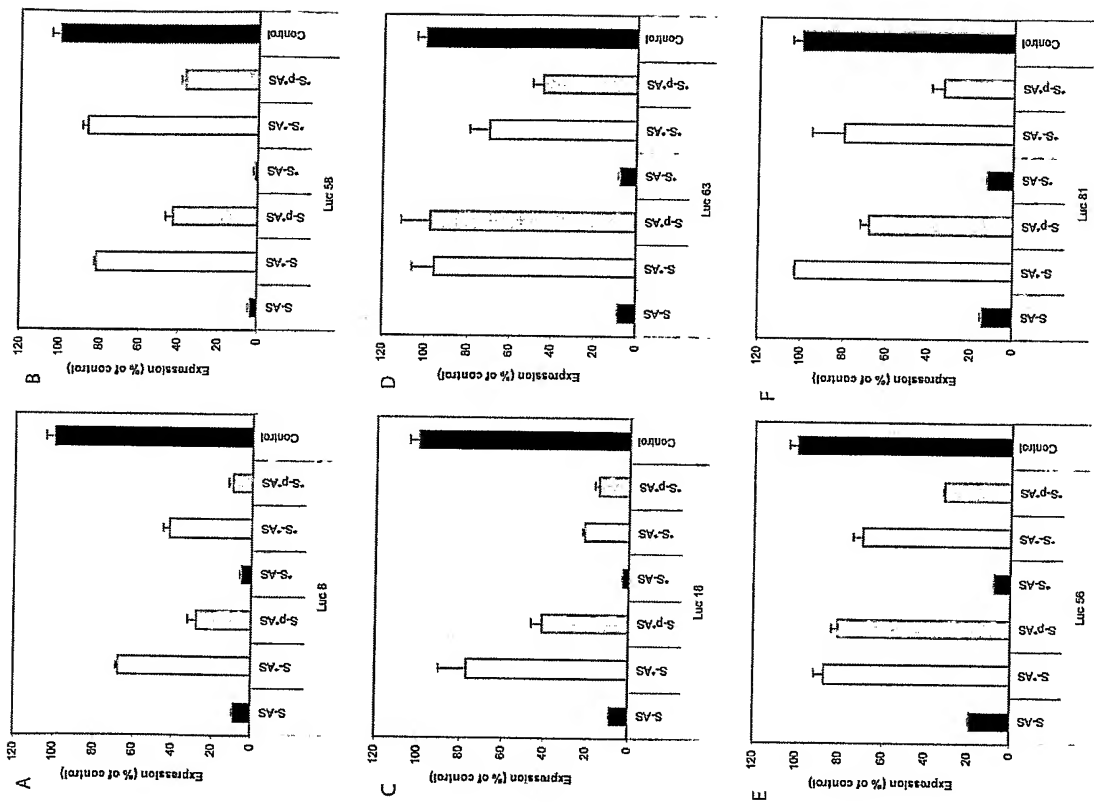


FIG. 6



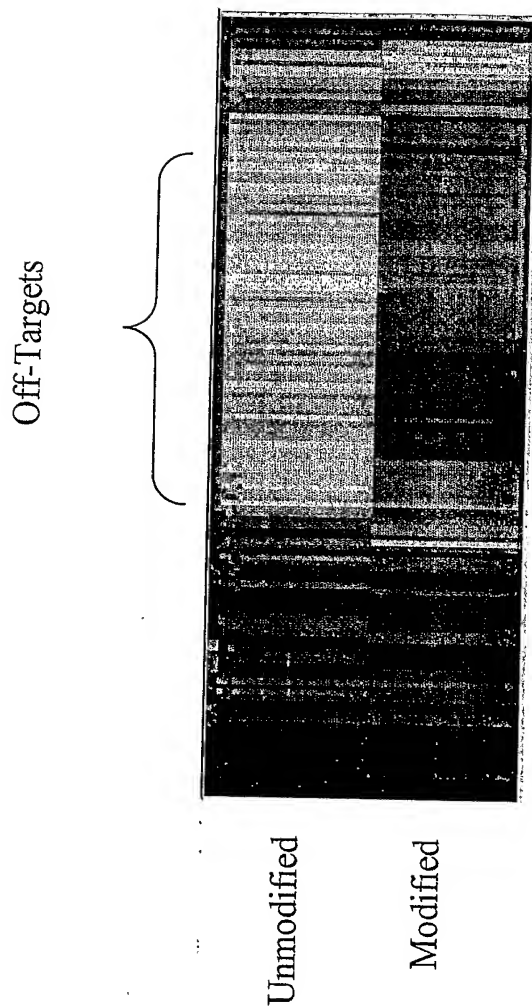
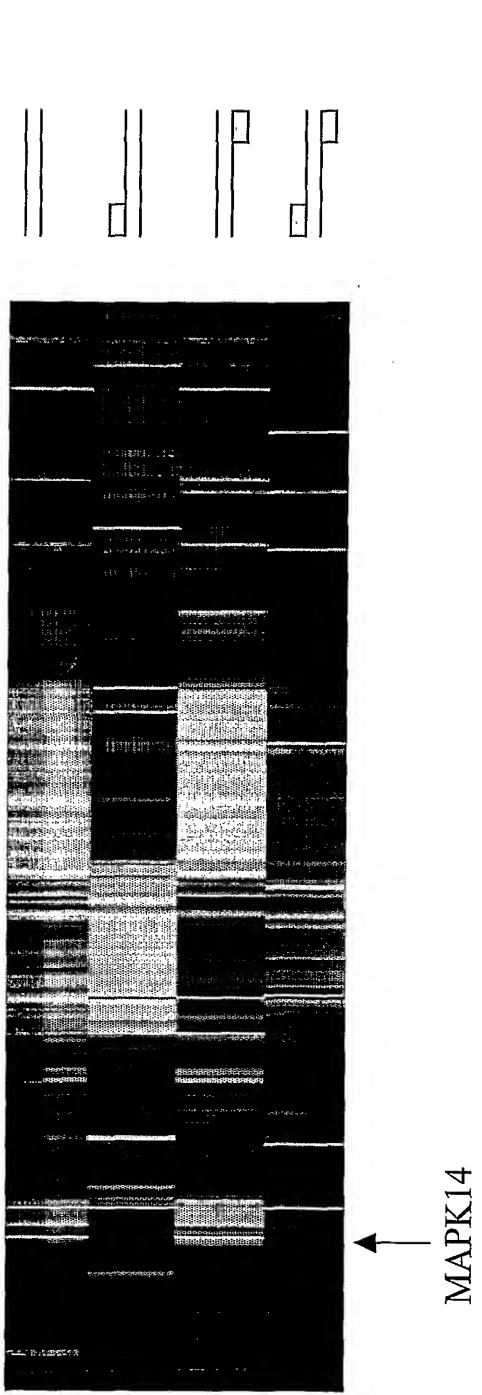


FIG. 7

A



B

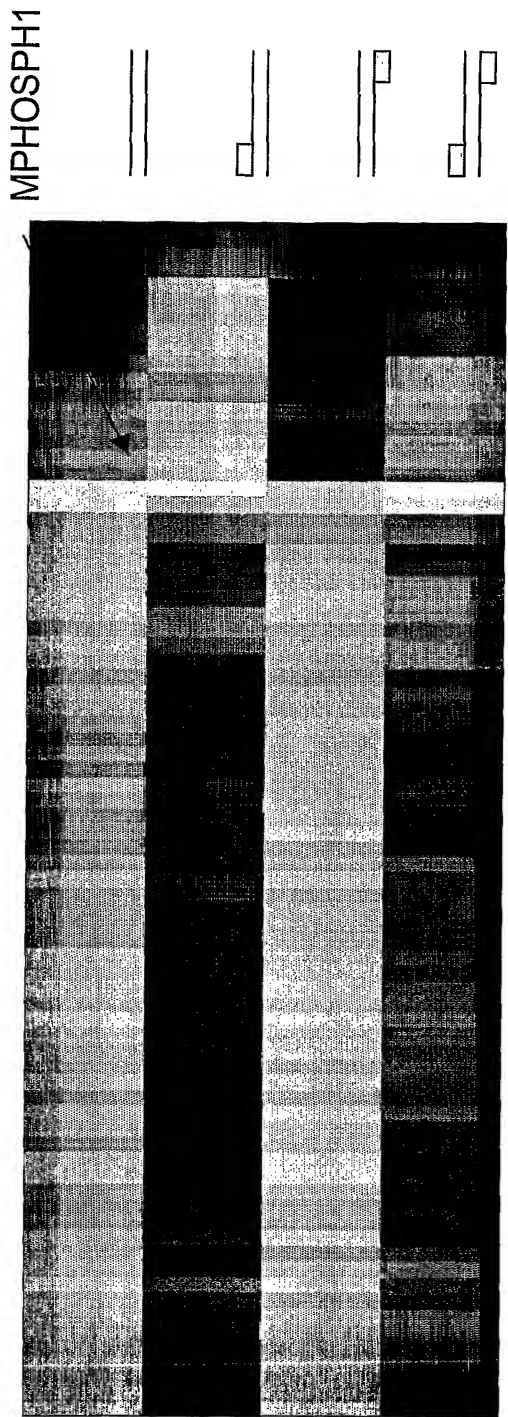
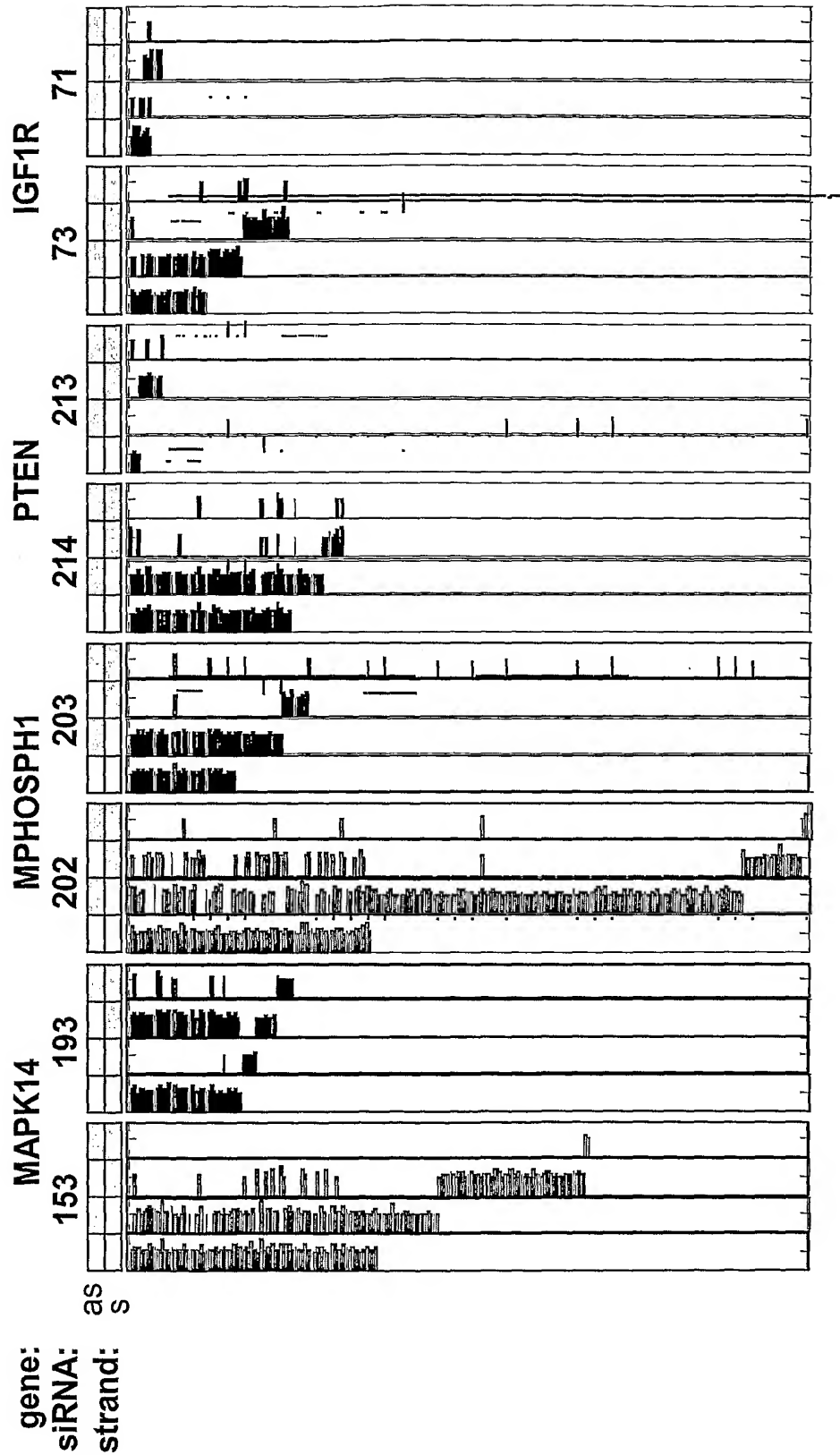


FIG. 8



MAPK14-153 Chem Walk

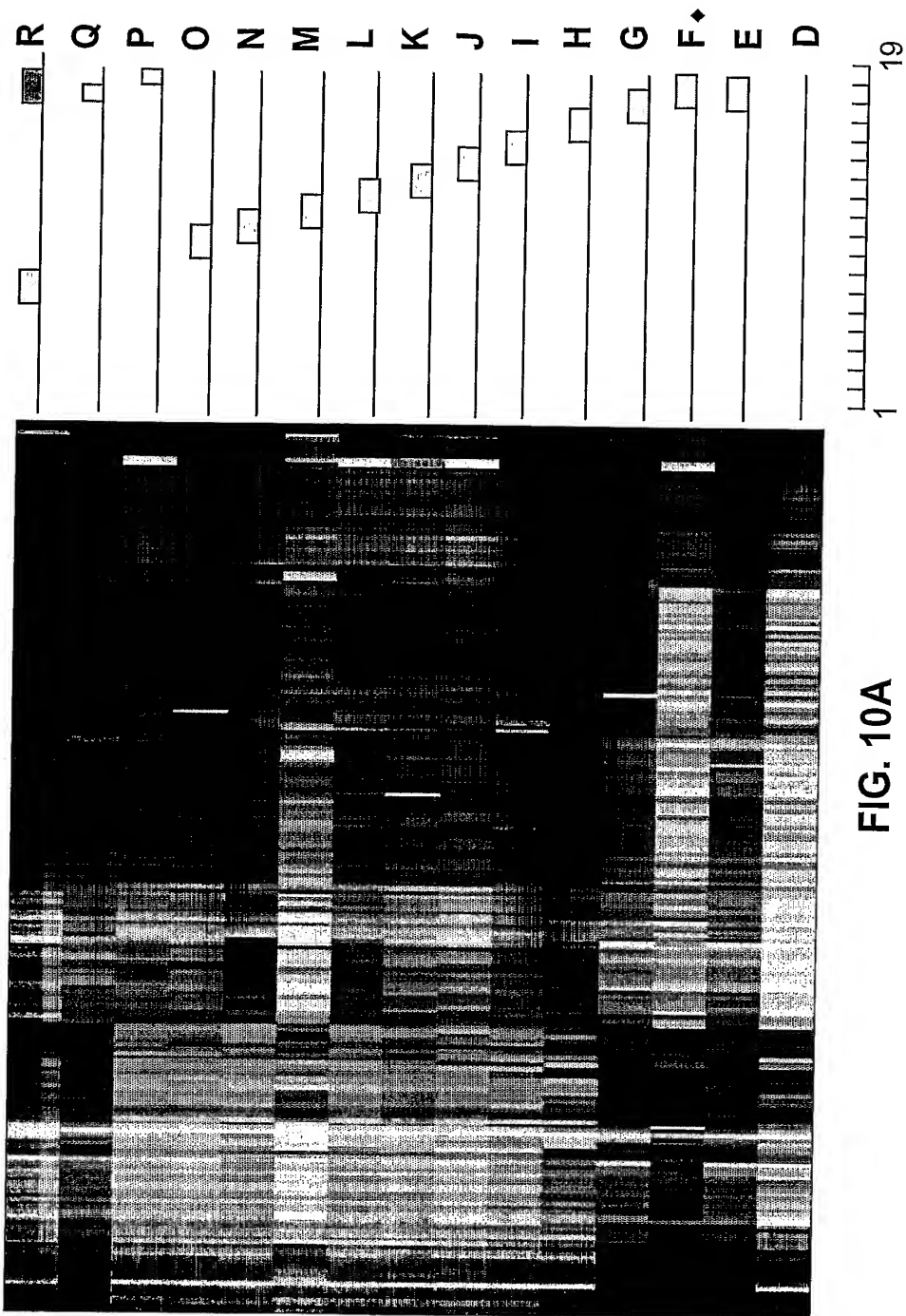


FIG. 10A

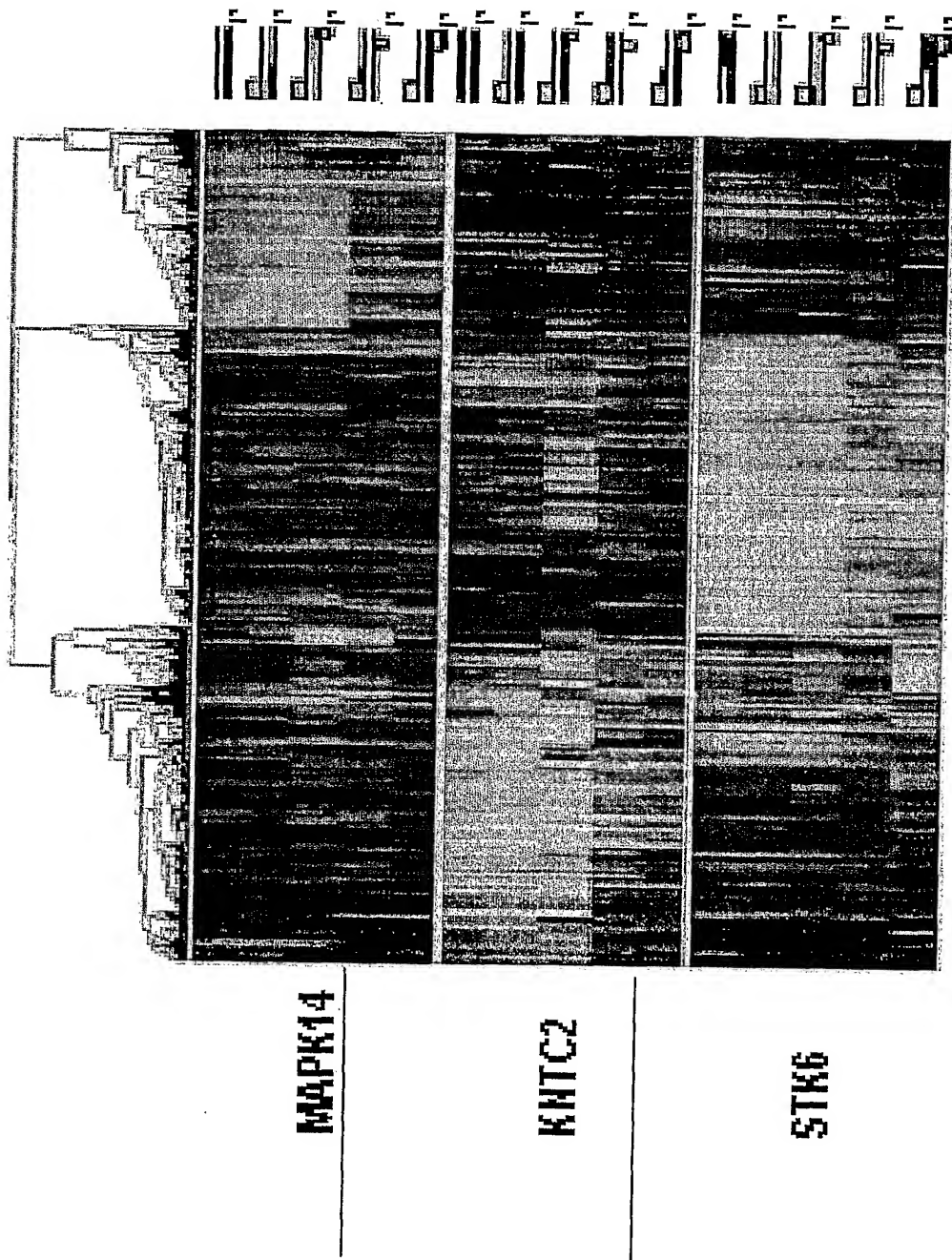


FIG. 10B

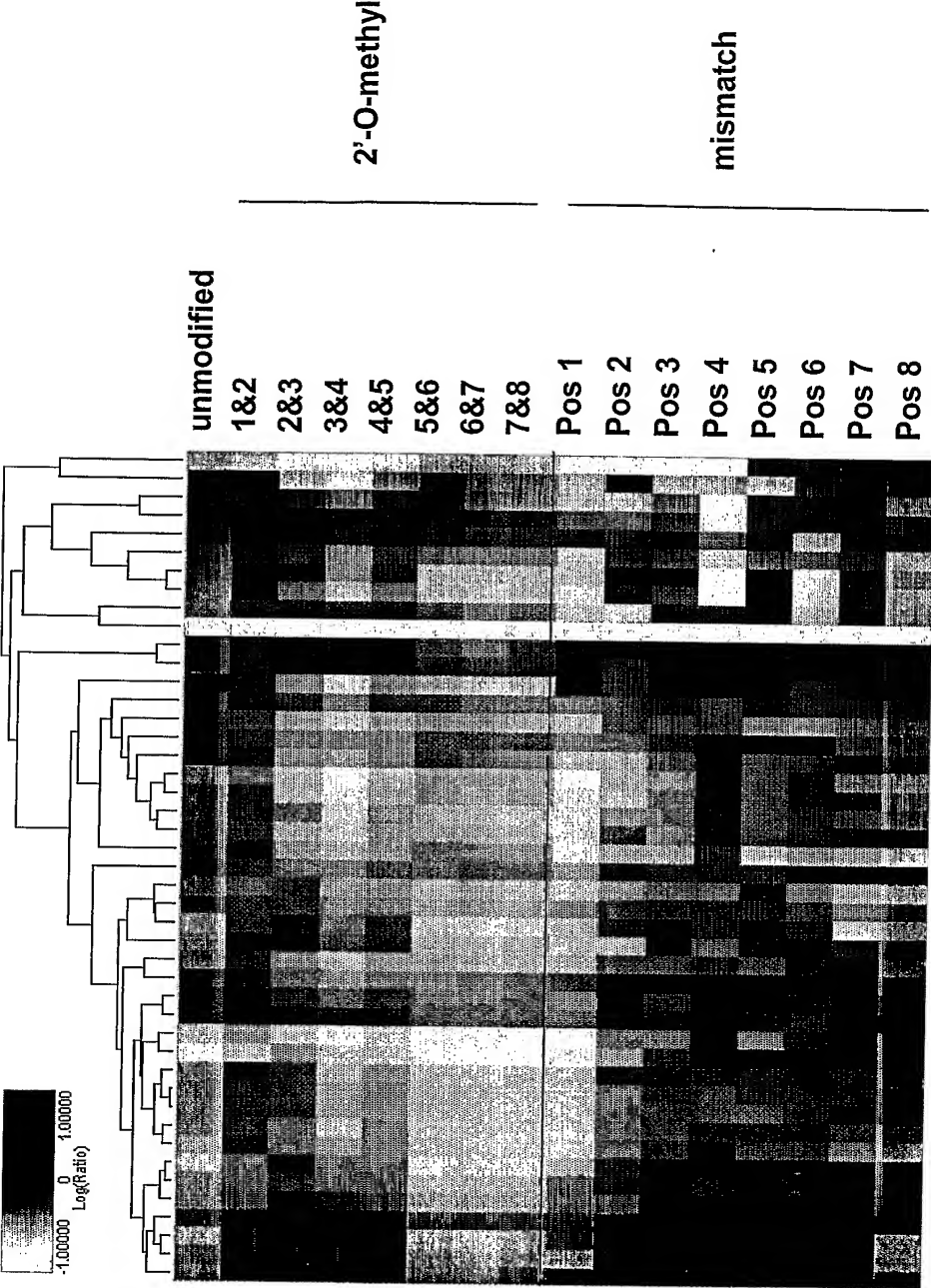


FIG. 11



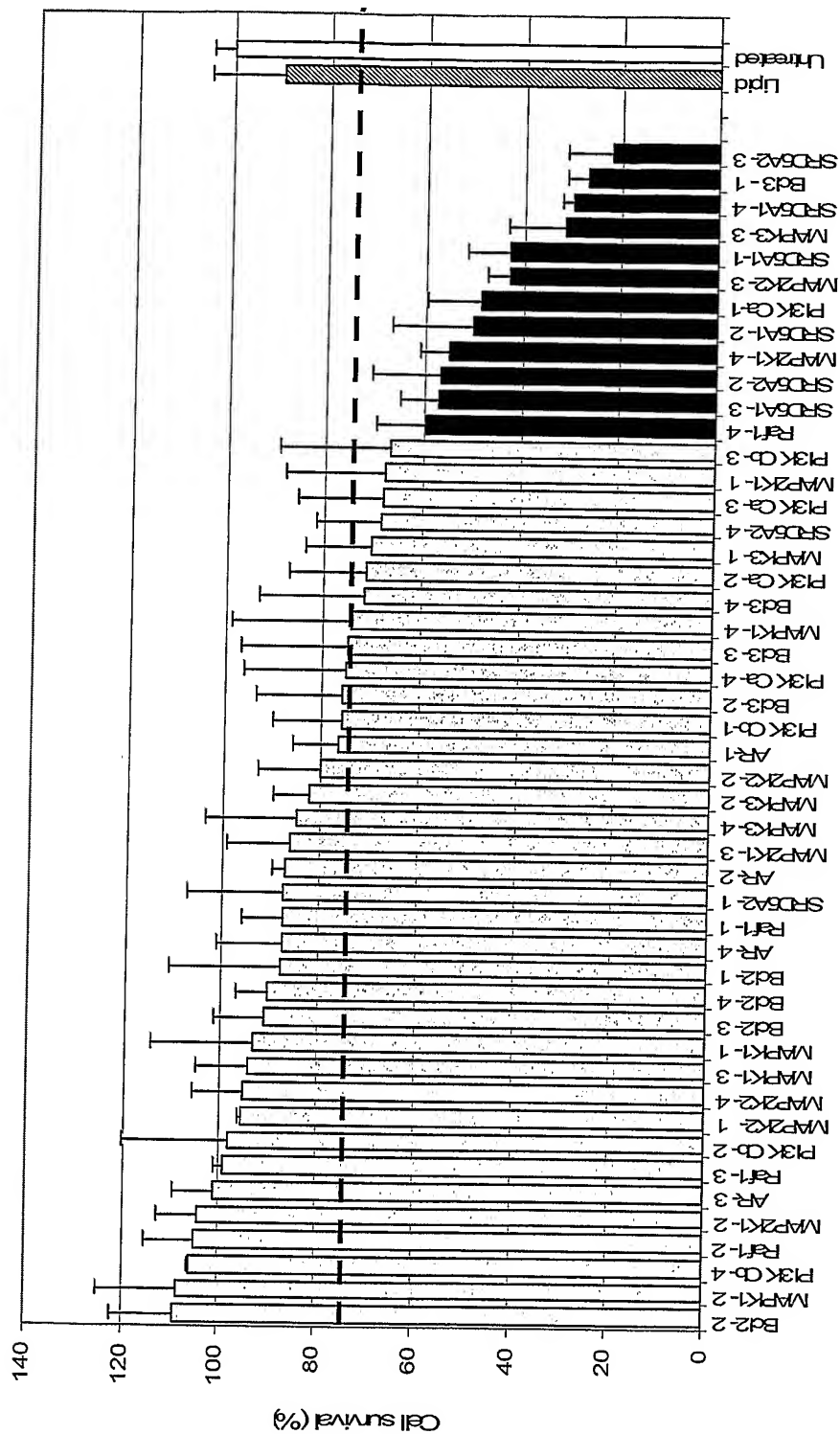


FIG. 12B



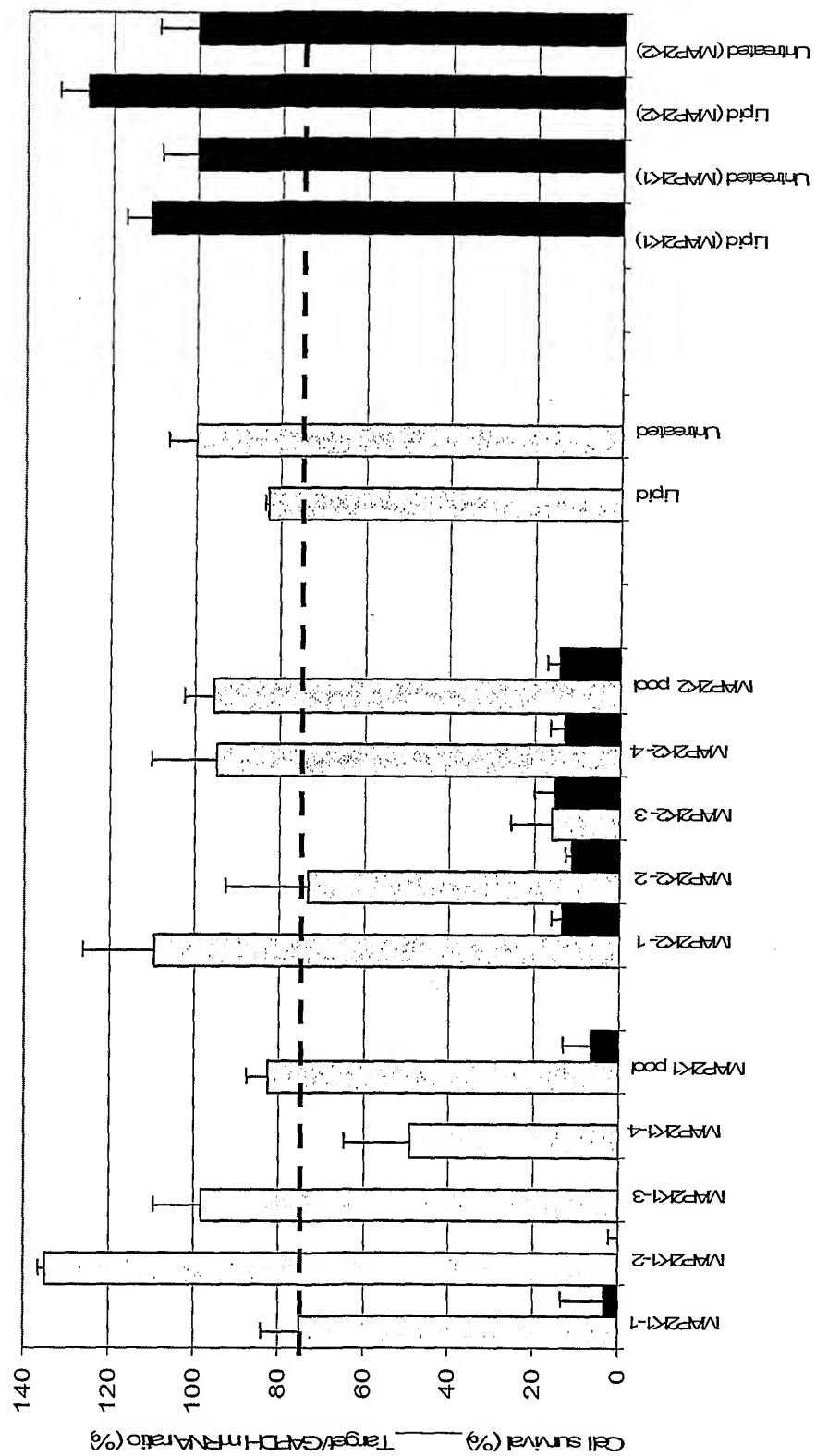


FIG. 12C

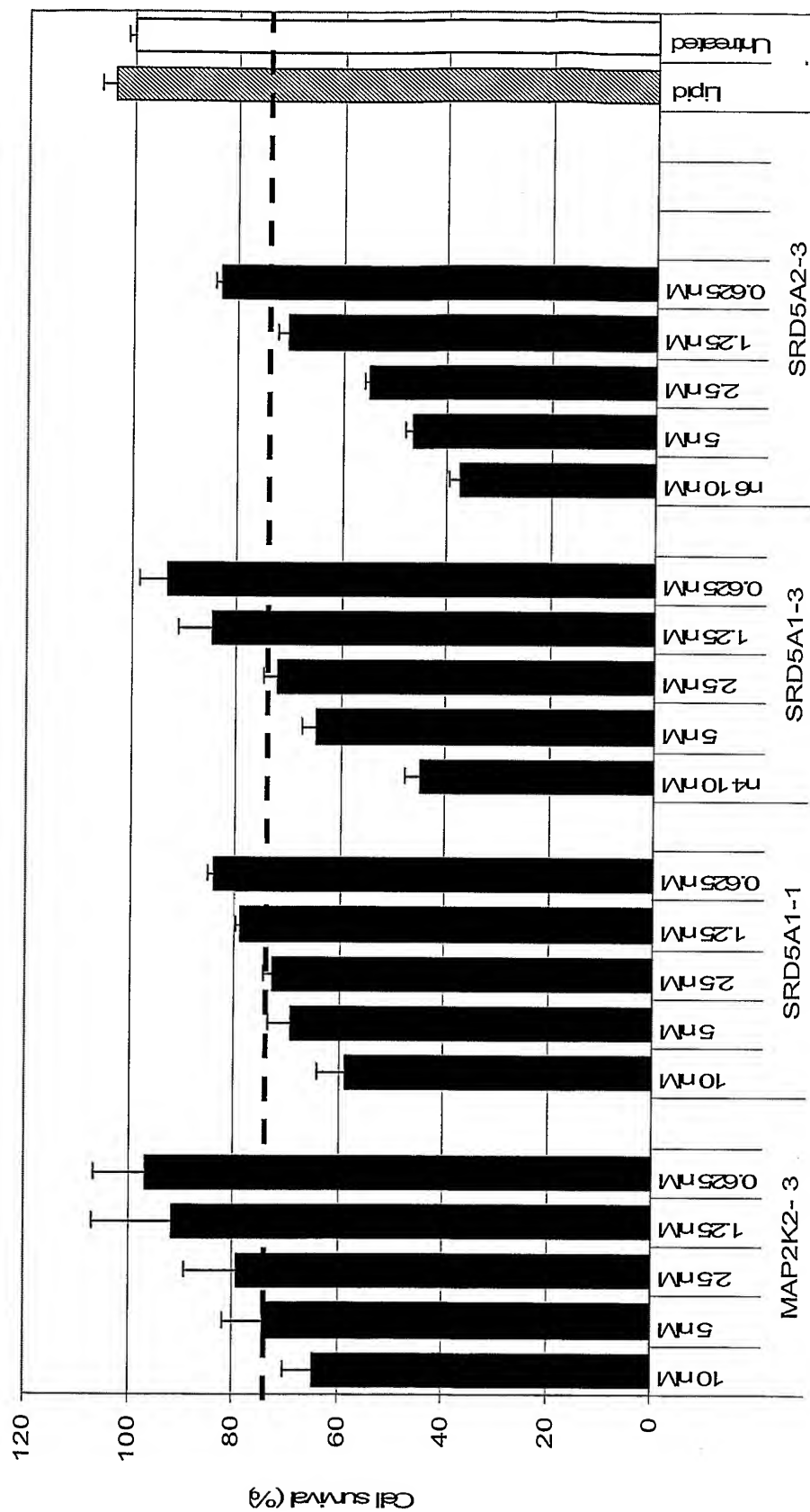


FIG. 12D

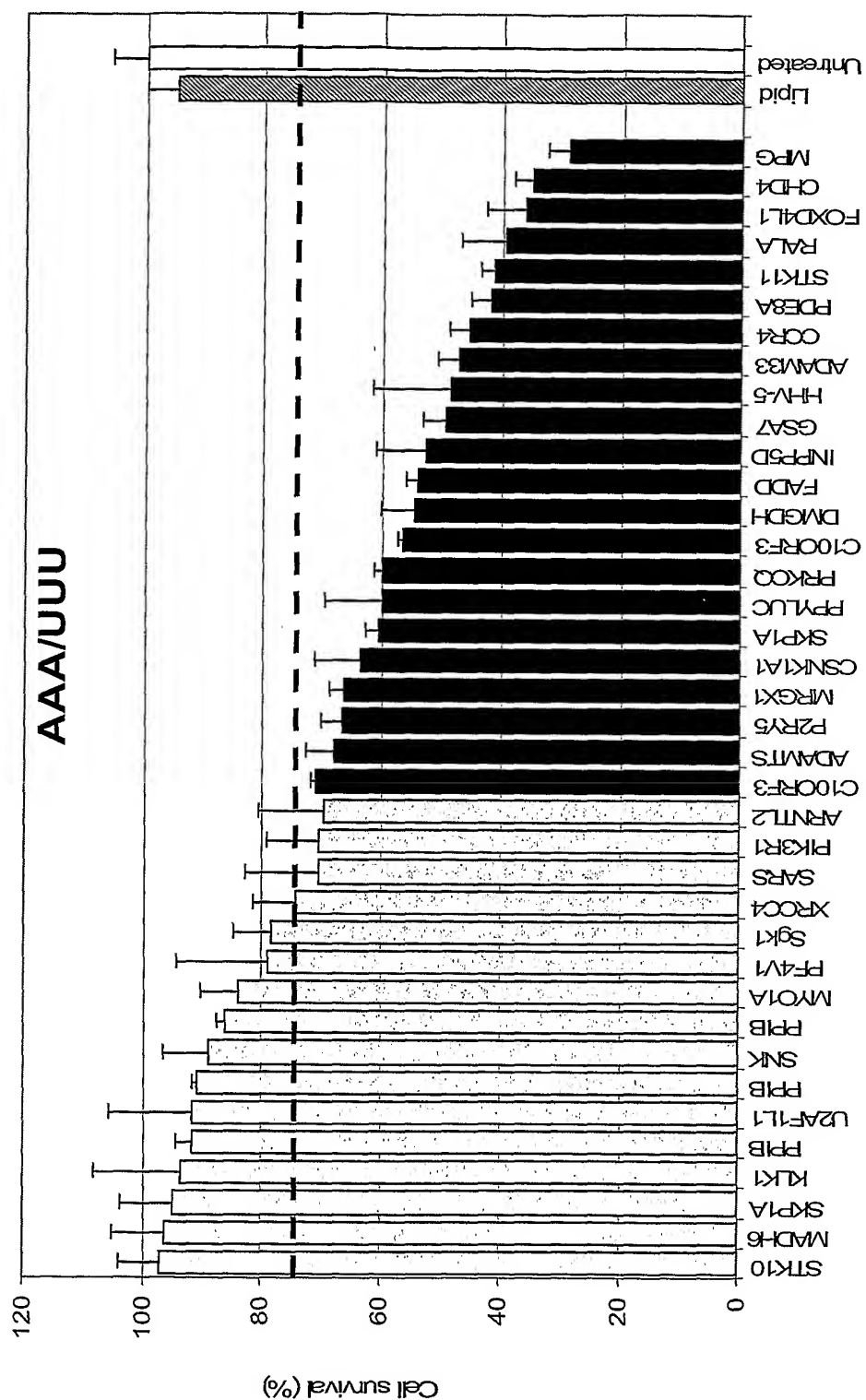
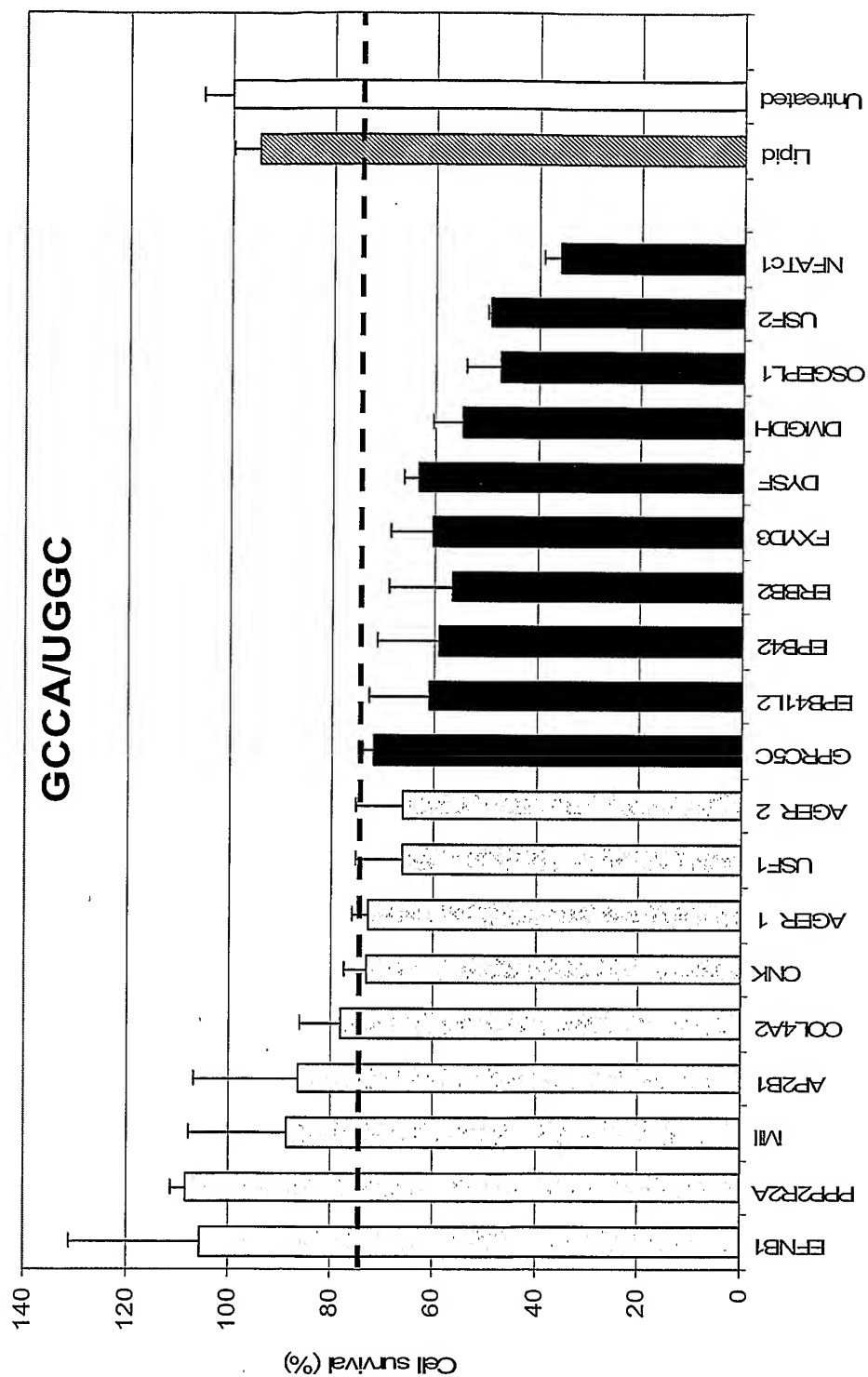


FIG. 13A



**FIG. 13B**

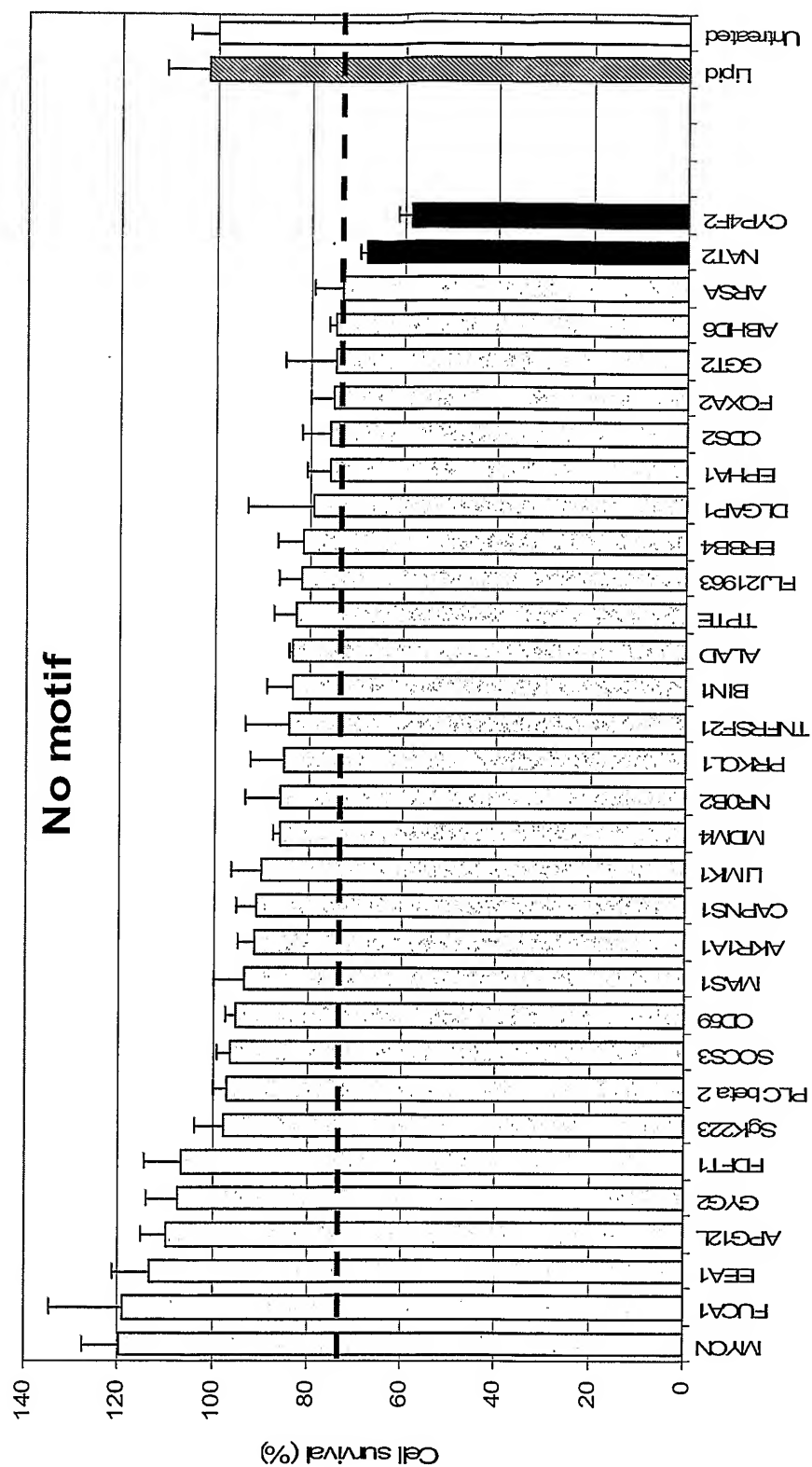
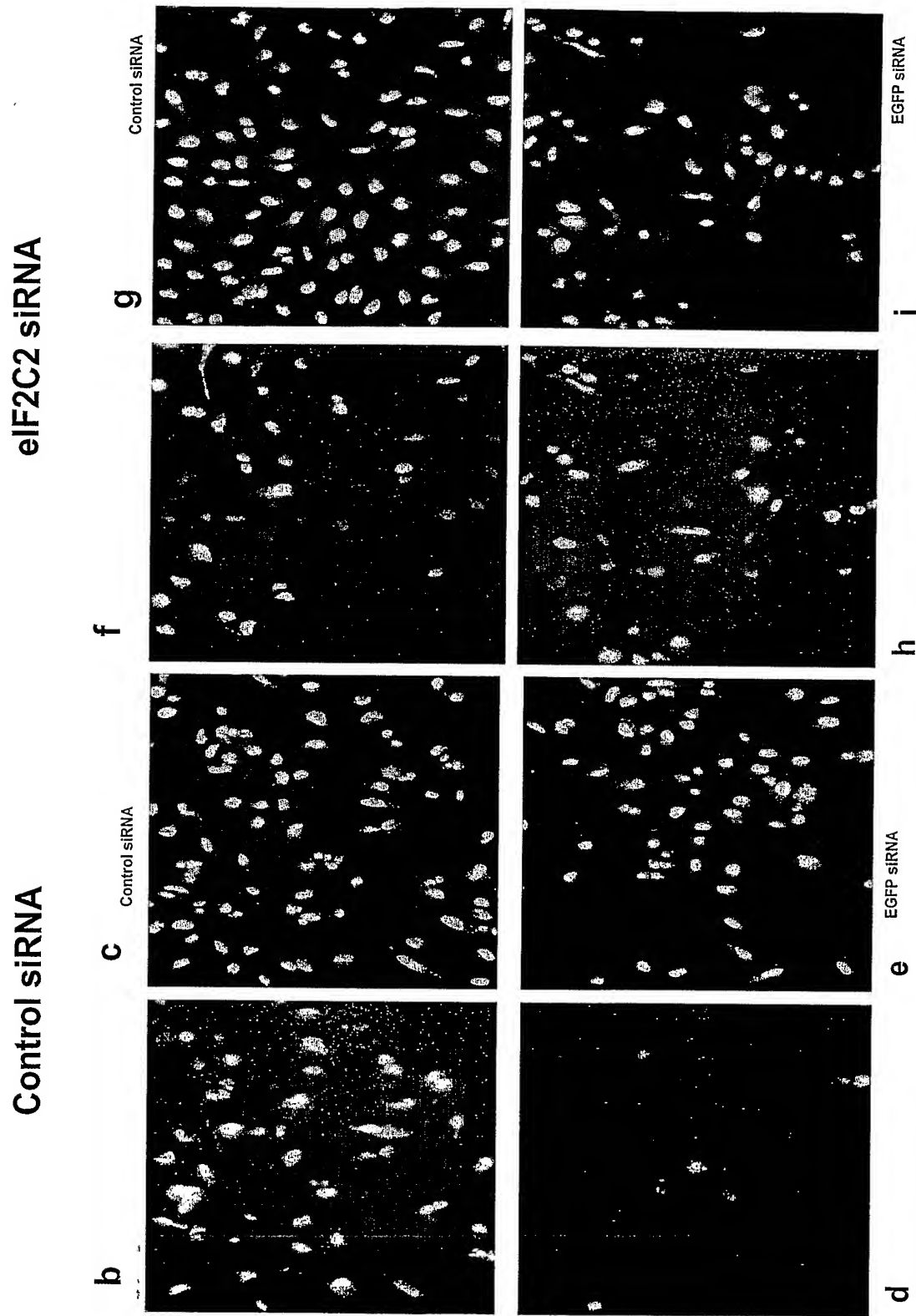


FIG. 13C





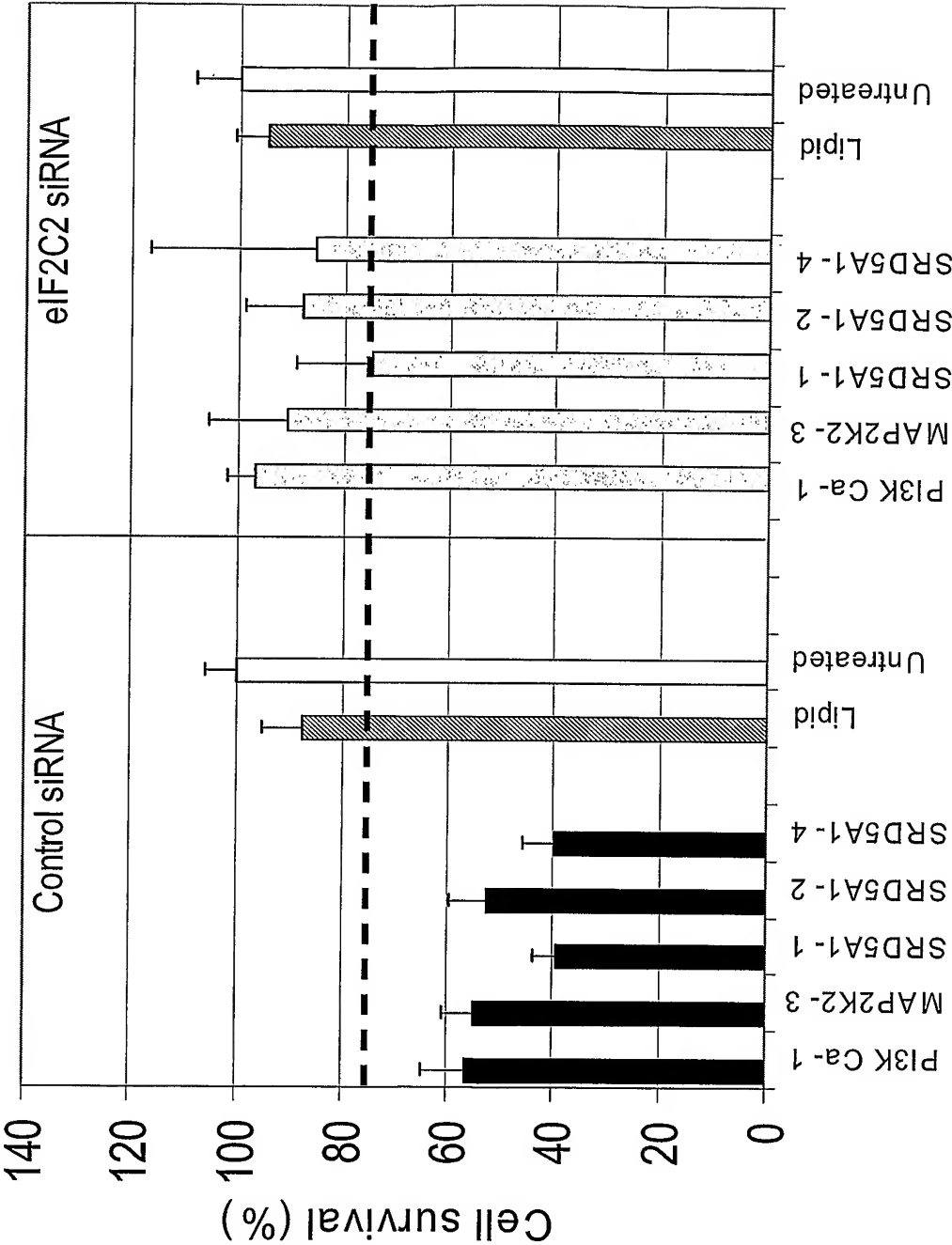


FIG. 14J



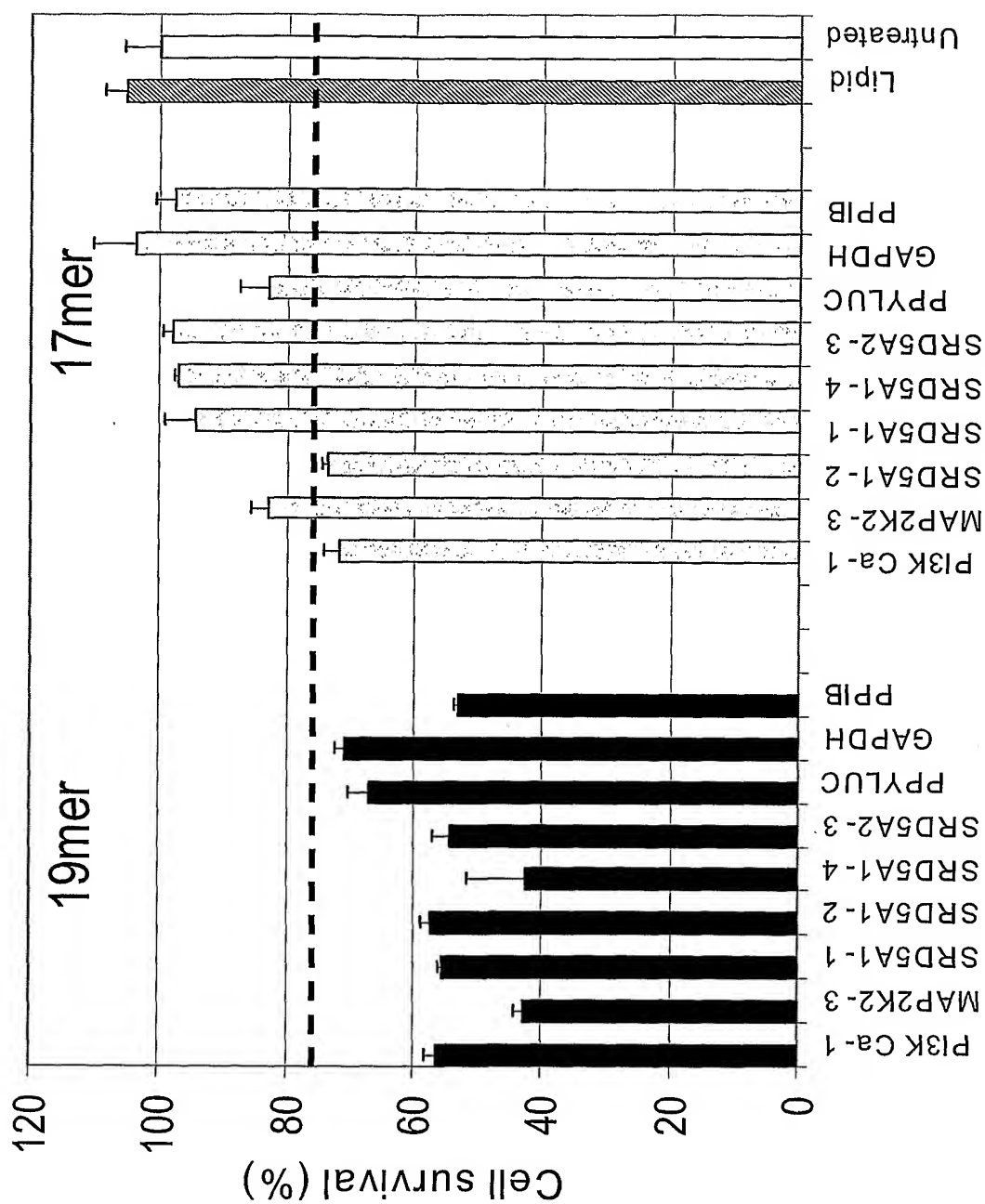


FIG. 14K

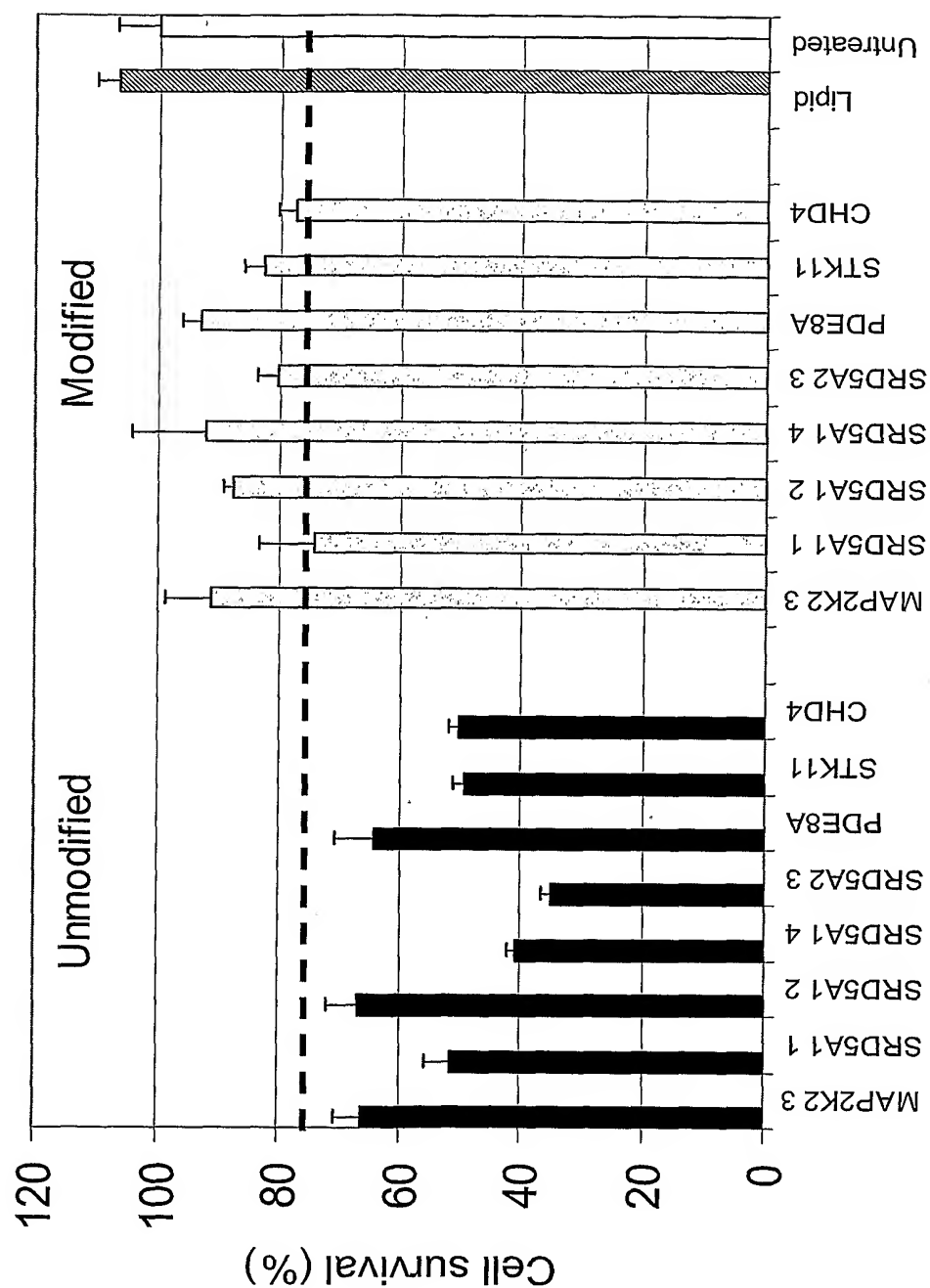


FIG. 14L

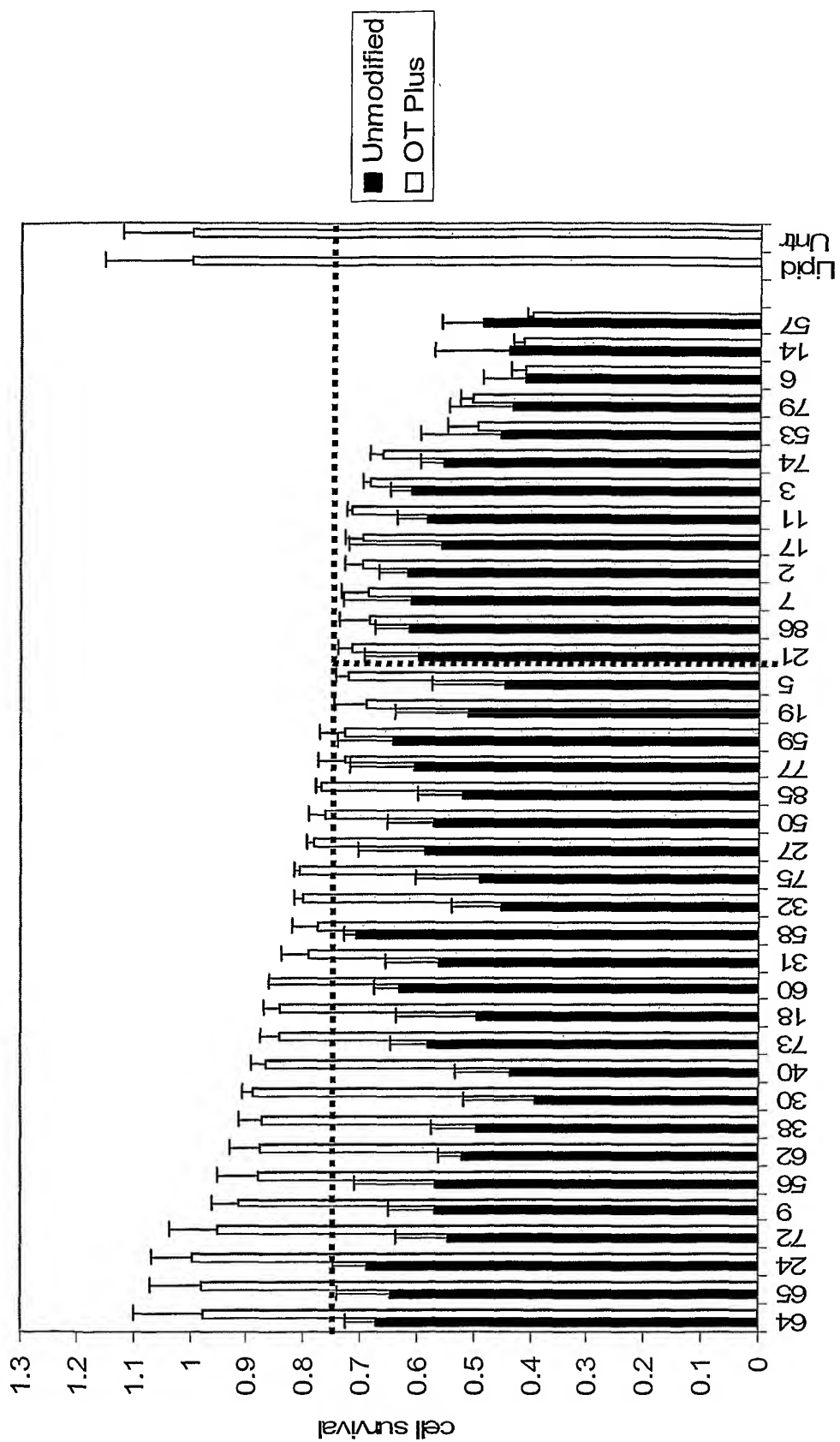


FIG. 14M

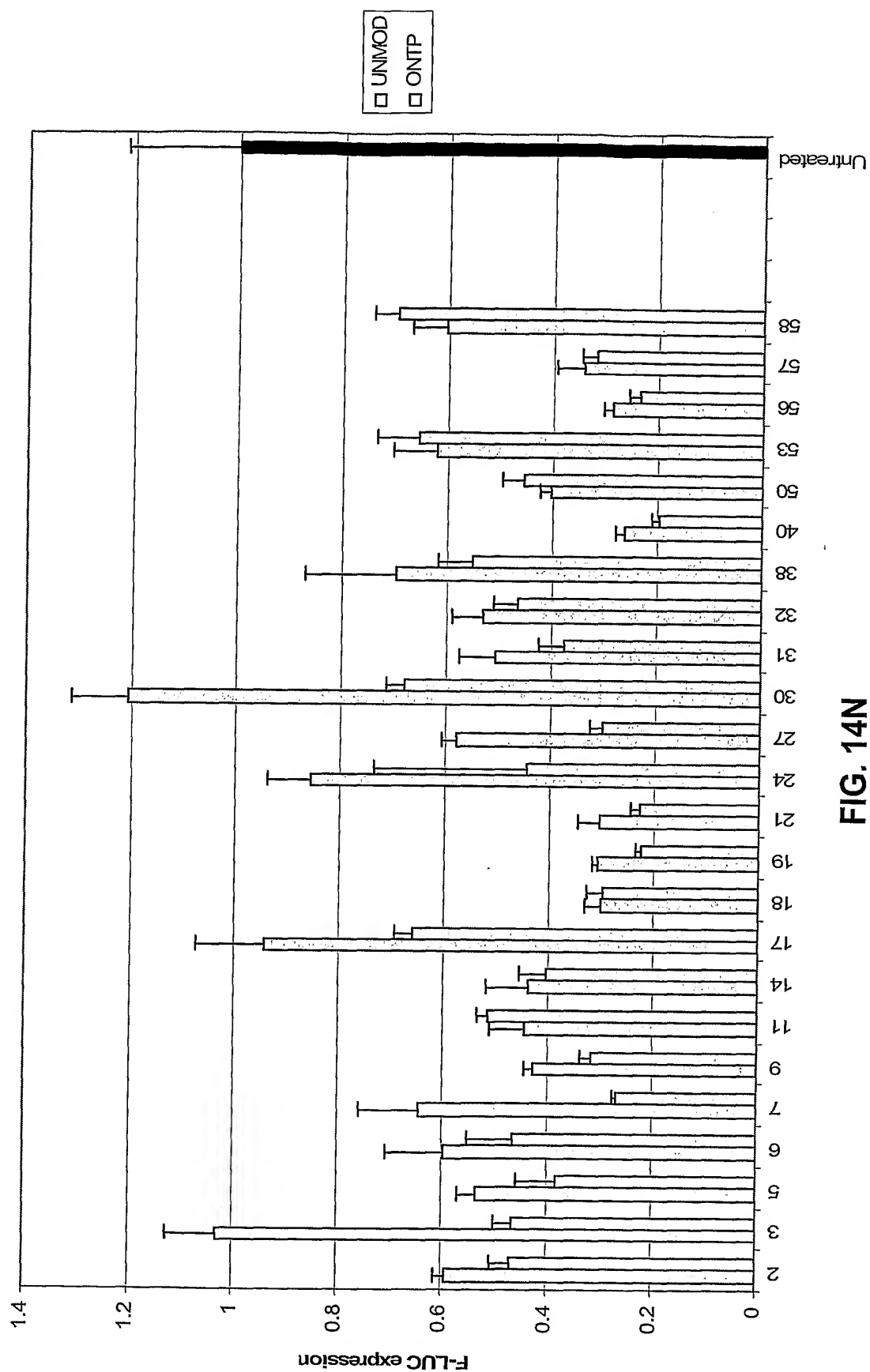


FIG. 14N

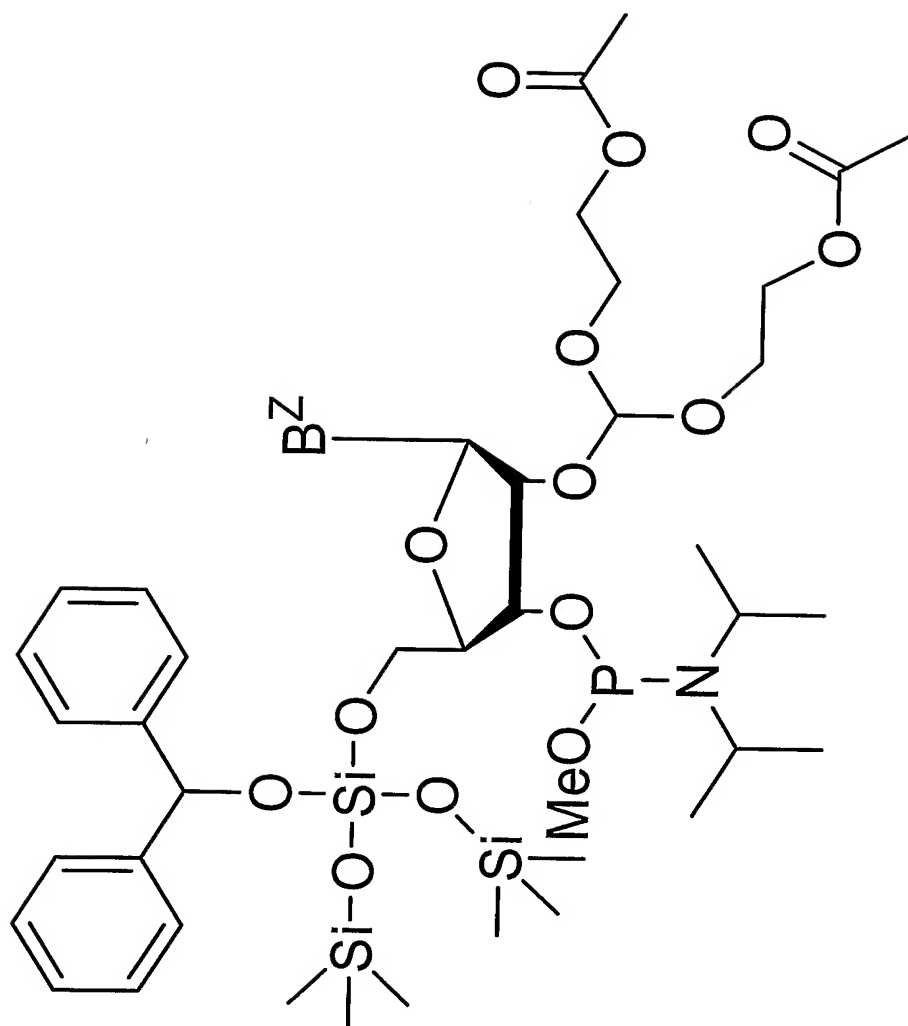


FIG. 15

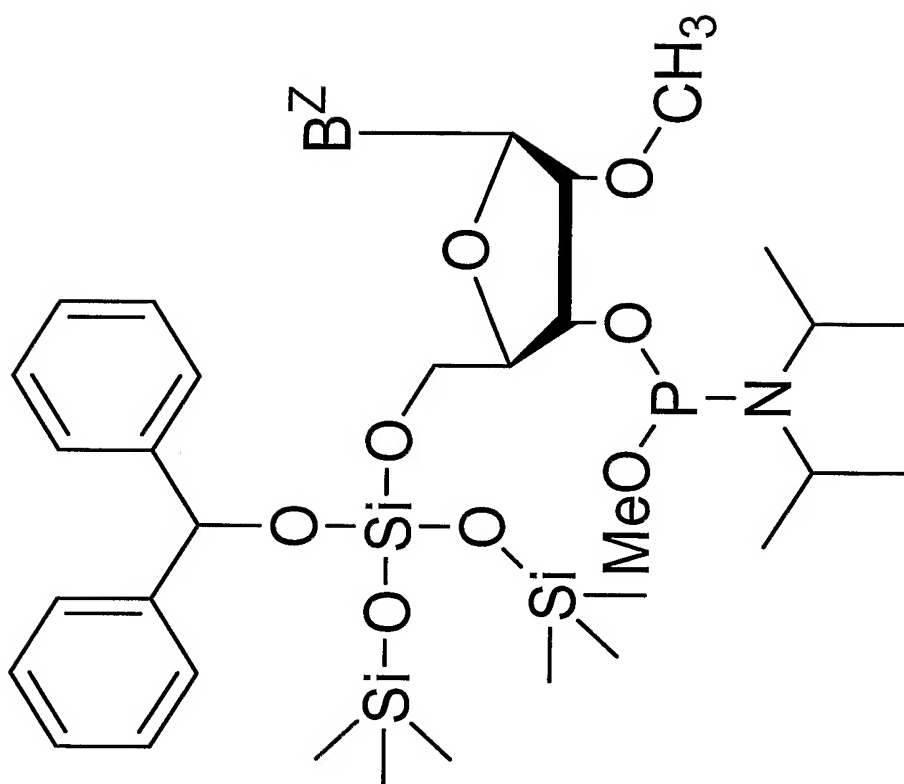


FIG. 16

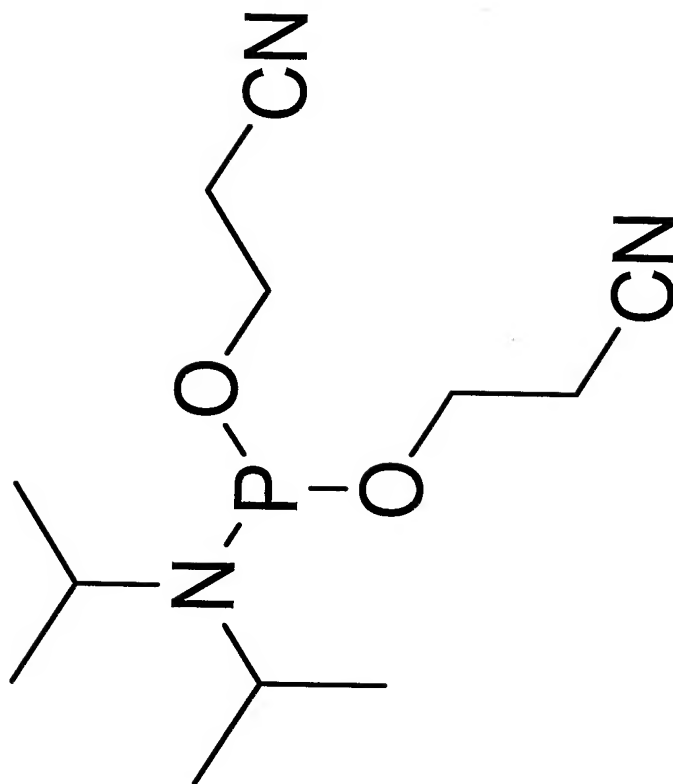


FIG. 17

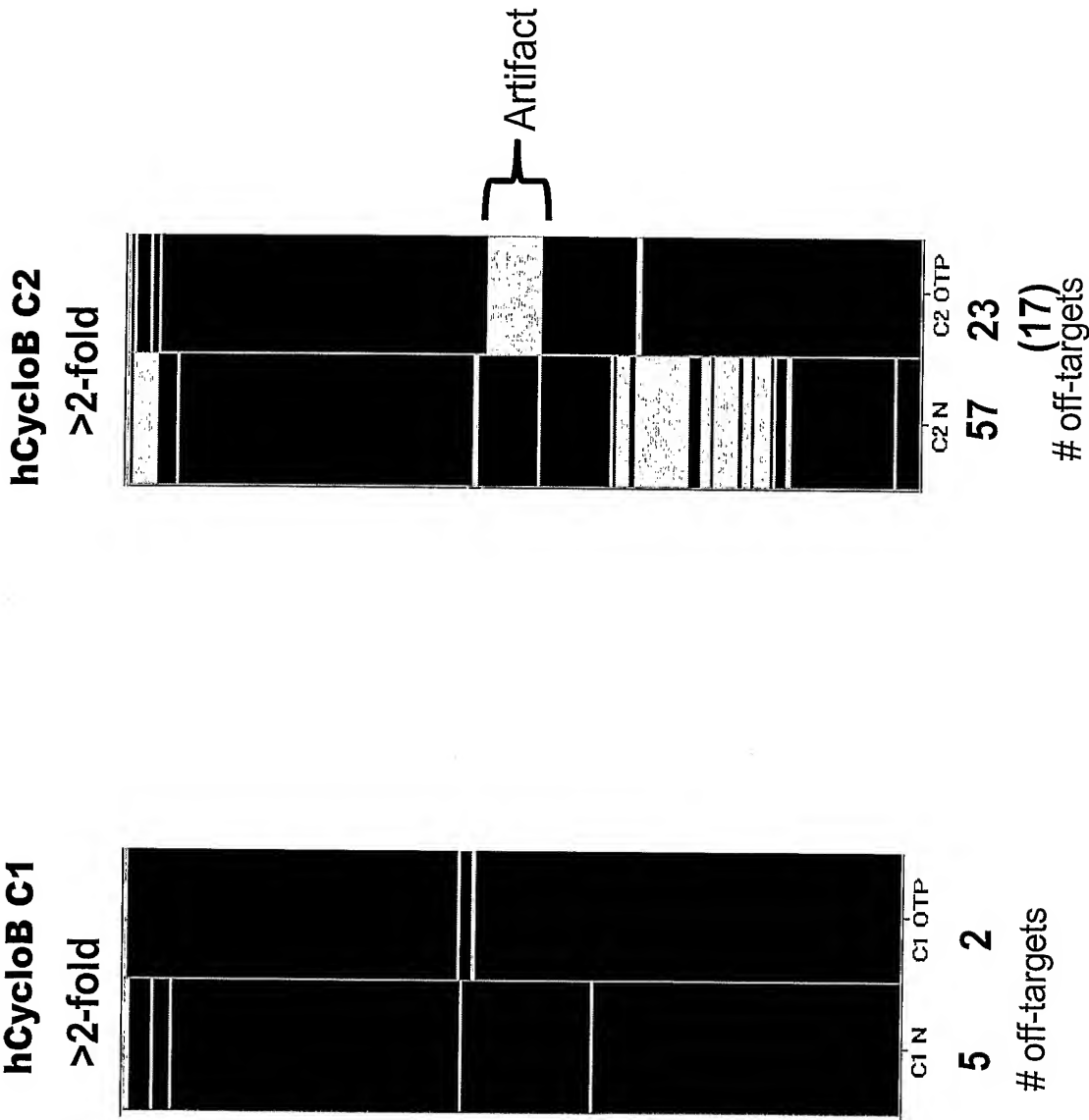
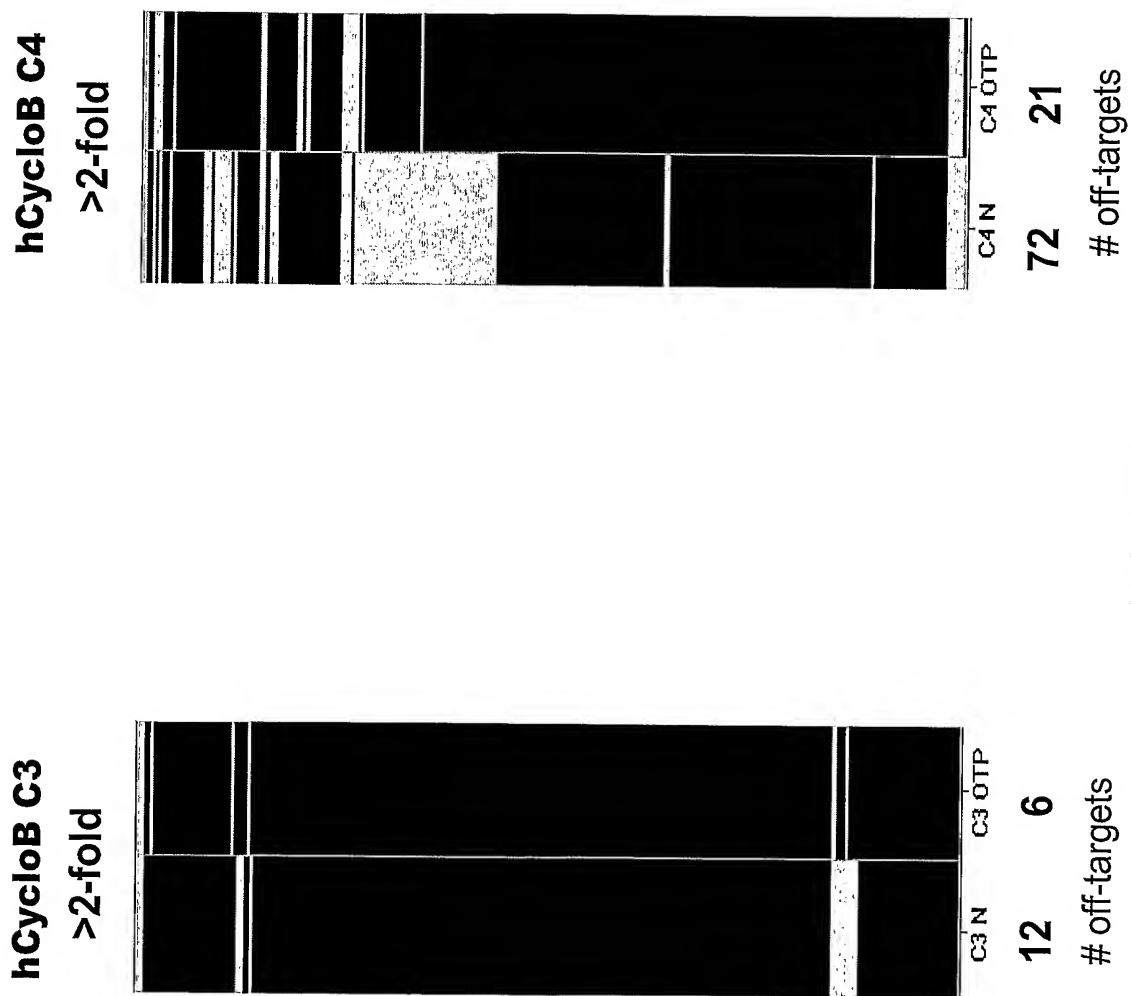
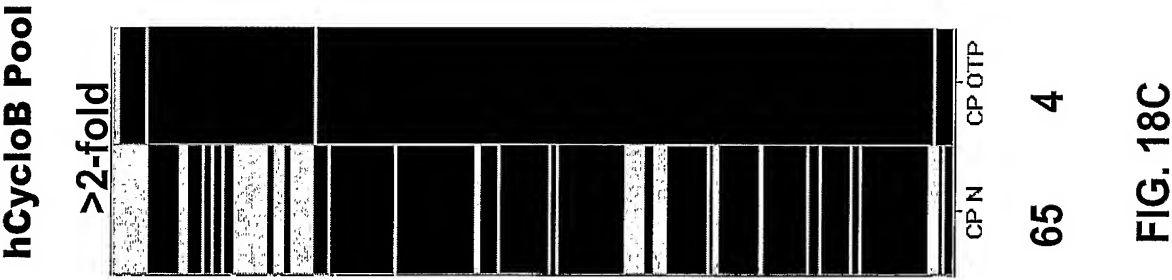


FIG. 18A





**FIG. 18B**



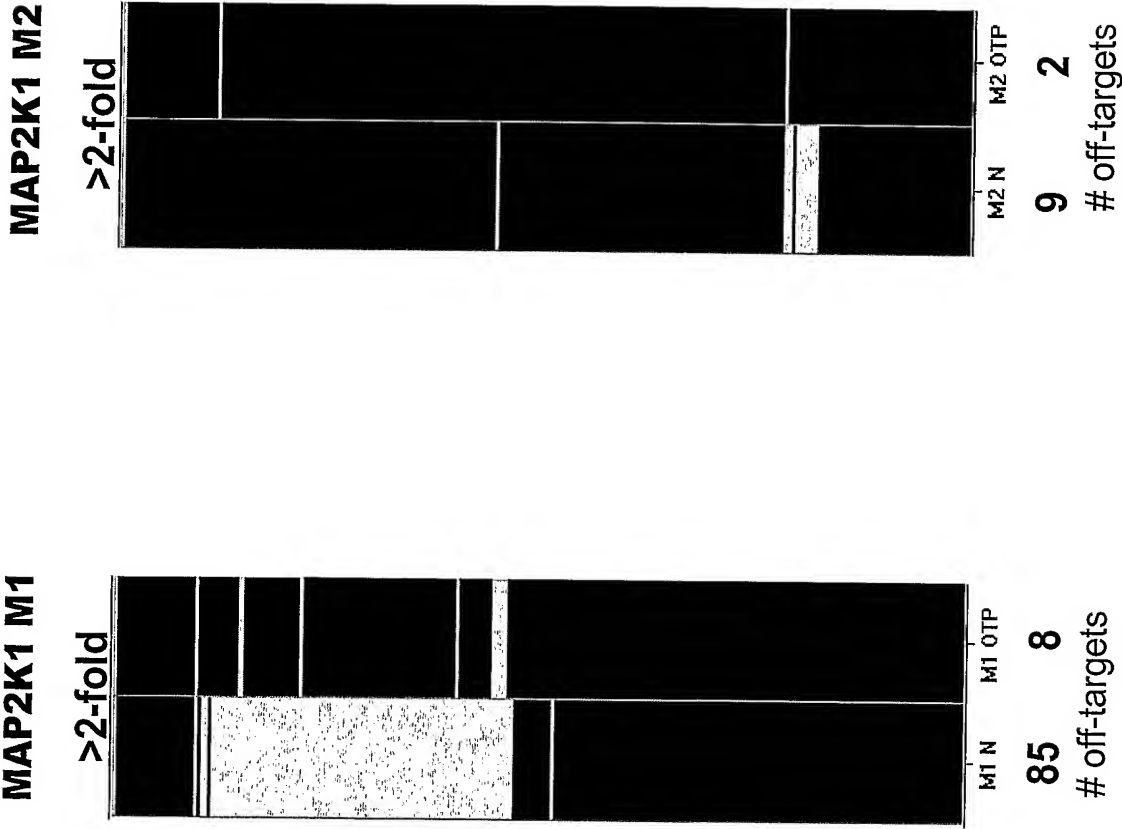


FIG. 18D

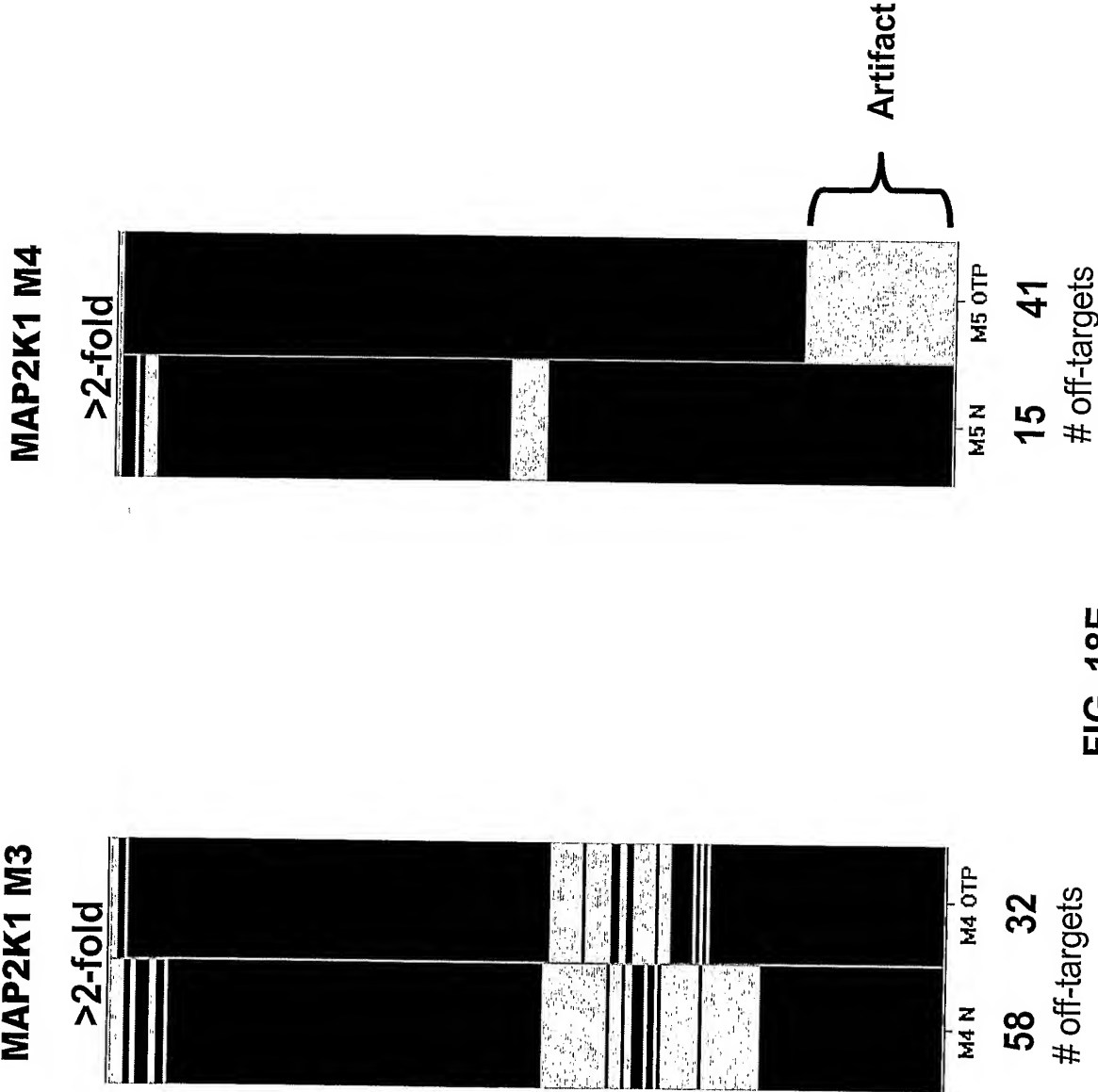
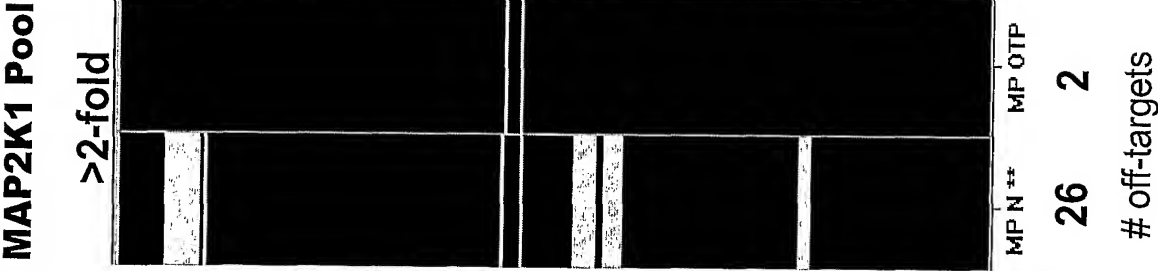


FIG. 18E



**FIG. 18F**

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 REYNOLDS, Angela  
 KHVOROVA, Anastasia  
 MARSHALL, William

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